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IMMUNITY IN GUINEA PIGS TO THE VIRUS OF VESICULAR STOMATITIS.

BY PERRIN H. LONG AND PETER K. OLITSKY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

It is known that the injection of immune serum into guinea pigs prevents generalization of the lesions but not the primary vesicles of foot-and-mouth disease. In studying a strain of the virus of vesicular stomatitis, a disease of horses closely related to foot-and-mouth disease of cattle,[1] we have found that the virus, when injected into guinea pigs, loses its original feeble power to produce the characteristic secondary lesions in the pad, and that only primary lesions arise after pad inoculation. Notwithstanding this fact, the virus receives a general distribution since it can be recovered, 48 hours after pad inoculation into guinea pigs, from the apparently normal tongue. On the other hand, when the virus is injected into the muscles or the skin (intradermal) elsewhere than in the pad, no local lesion whatever follows, and 10 days after the inoculation it is found that the pigs are immune to reinoculation.

In the preliminary experiments, no attempt was made to titrate the strength of the virus, because present methods are crude, and once the infectivity of a given sample of virus has been determined, it is impossible to estimate the rate of its deterioration. Guinea pig pad vesicle fluid, obtained 24 to 48 hours after inoculation, diluted 1:10 and 1:20 with phosphate buffer at a pH of 7.5; and filtered through a Berkefeld "V" candle, was employed in the following experiments. The immune serum was obtained from guinea pigs 10 to 14 days after inoculation.

Neutralization of the virus *in vitro* was first undertaken. 1 cc. of a 1:10 dilution of the virus was added to 1 cc. of immune serum and the contents of the tubes thoroughly mixed. The tubes were kept at

[1] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx, 147; Olitsky, P. K., *J. Exp. Med.*, 1927, xlv, 969.

room temperature for one minute, one hour, and 24 hours respectively, at which times the virus-serum mixture was injected into the pads of normal guinea pigs. Normal guinea pig serum was used as a control. Lesions appeared only in the animals treated with normal serum. After 10 days the pigs inoculated with immune serum and virus were retested by pad inoculation. All developed typical lesions.

Next, the pads of 4 normal guinea pigs were infiltrated with immune serum and the pads of 2 with normal guinea pig serum. One hour later the pads of 2 of the guinea pigs treated with immune serum, and those of one pig with normal serum were injected with a 1:20 dilution of the virus. The remaining 3 pigs were similarly inoculated with a 1:20 dilution of the virus on the following day. Lesions appeared only in the pads infiltrated with normal serum. Ten days later, all animals were reinoculated in the pads with active virus, and only the immune serum group developed typical vesicles.

Following this experiment, we determined the protection given by the intramuscular injection of immune serum, succeeded by intracutaneous or intramuscular inoculation of the virus. 0.5 cc. of immune serum was injected into the right thigh muscles of 8 normal guinea pigs. One hour later 2 animals were injected in the pads with a 1:10 dilution, and 2 with 0.5 cc. of a 1:20 dilution of the virus in the left thigh muscles; 24 hours later this procedure was repeated with the 4 remaining guinea pigs. Typical lesions appeared in the pads of the 4 animals inoculated in this tissue, while those receiving the virus intramuscularly revealed no manifest lesions. On retesting 10 days later with active virus, the animals inoculated intramuscularly developed typical lesions.

The conclusions which we draw from these experiments are: (1) that the virus generalizes through the body, although it induces no visible changes; (2) that following single intramuscular or intracutaneous inoculation of living virus at sites other than the pads, although no manifest lesions occur, a solid immunity results; (3) that a certain degree of neutralization of vesicular stomatitis virus *in vitro* and *in vivo* results from the addition of the serum of immune animals; (4) that no immunity results from the injection of neutralized virus.

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MULTIPLICATION DU VIRUS DE LA STOMATITE VÉSICULAIRE DU CHEVAL DANS DES CULTURES DE TISSUS.

PAR ALEXIS CARREL, PETER K. OLITSKY, ET PERRIN H. LONG.

(*Des laboratoires de l'Institut Rockefeller.*)

Le but des expériences résumées dans cette note était de déterminer dans quelles conditions le virus de la stomatite vésiculaire du Cheval peut se multiplier dans des cultures de tissus. Le virus était suspendu dans une solution de phosphate ou dans de la solution de Tyrode de *p*H 7,5, et on déterminait sa virulence par inoculation à des Cobayes. Après avoir été dilué plus ou moins avec de la solution de Tyrode, il était mis au contact, pendant une période variant de 1 heure et demie à 24 heures, avec de la moelle osseuse de Cobaye ou avec de la pulpe de fœtus du même animal. Au bout de ce temps, les fragments de tissus étaient cultivés dans des flacons D-3 contenant un milieu solide composé d'un volume de plasma de Cobaye et d'un volume de solution de Tyrode. A la surface du coagulum était injecté un milieu nutritif composé de sérum de Cobaye, s'il s'agissait de cultures de moelle osseuse, et de suc d'embryon de Poule, dans les cultures de pulpe fœtale. Les cultures étaient placées dans une étuve à 39°. Tous les deux ou trois jours, elles étaient lavées avec de la solution de Tyrode et on remplaçait le liquide par un milieu nutritif frais. Au bout de 7 ou 9 jours, on retirait les tissus des flacons, on les broyait dans une solution salée, et on les inoculait à des Cobayes dans les pattes postérieures. Cinq expériences furent faites.

Expérience 1.—N° 478-H, 19 avril 1927. Virus filtré actif à 1 p. 10,000. On le dilua à 1 p. 50 avec de la solution de Tyrode. Dans 5 c.c. de la suspension, on plaça 1 c.c. de pulpe de foie, de cerveau, et de poumon d'un fœtus de Cobaye. Après un séjour de 3 heures dans le réfrigérateur, les tissus furent cultivés dans des flacons D-3. Au bout de 10 jours, on examina les tissus sans y déceler la présence du virus. Aucun des animaux auxquels on injecta les cultures ne fut immunisé.

Expérience 2.—N° 580-H, 10 mai 1927. Virus filtré actif à 1 p.

1.000.000. Le virus fut dilué à 1 p. 50 avec de la solution de Tyrode. On mélangea 1 c.c. de pulpe de jeune fœtus de Cobaye à 1 c.c. de virus dilué de 1 p. 50.000 à 1 p. 500.000. Après quatre heures de contact dans le réfrigérateur, les tissus furent cultivés de la façon habituelle. Au bout de 7 jours, l'examen des cultures montra que le virus avait disparu. Aucun des animaux inoculés avec les cultures ne fut immunisé.

Expérience 3.—N° 1307-H, 2 novembre 1927. Virus filtré actif à 1 p. 800, et inactif à 1 p. 8.000. Le virus fut dilué pour 40 volumes de solution de Tyrode. Des fragments de moelle osseuse de Cobaye furent mis au contact du virus, dans un réfrigérateur, pendant des périodes variant de 1 heure et demie à 24 heures. Au bout de ce temps, on cultiva les tissus dans du plasma de Cobaye. L'examen des cultures, au bout de 7 et 8 jours, montra que le liquide était actif à une dilution de 1 p. 1.000.000. On n'employa pas de dilutions plus élevées. Une des cultures des tissus qui avaient été en contact avec le virus pendant 3 heures et demie ne montra, au bout de 13 jours, aucune activité, même à la dilution de 1 p. 1.000.

Expérience 4.—N° 296-H, 1er mai 1927. Virus filtré actif à 1 p. 10 millions. De la pulpe de rate, de foie, de poumon, et de cerveau de fœtus de Cobaye fut mise au contact du virus à des dilutions variant de 1 p. 1.000 à 1 p. 10.000, pendant 24 heures, dans le réfrigérateur. Ensuite, cette pulpe fut cultivée dans des flacons de la façon habituelle. Au bout de 7 jours, le liquide des cultures était actif à une dilution de 1 p. 100 millions.

Expérience 5.—N° 370-H, 28 mars 1927. Virus filtré actif à une dilution de 1 p. 100.000. Des fragments de moelle osseuse furent placés dans des dilutions du virus à 1 p. 10.000, à 1 p. 100.000, et à 1 p. 1.000.000, pendant trois heures. Puis ils furent cultivés de la façon habituelle pendant 7 jours. Le virus contenu dans les cultures était, à ce moment, actif à une dilution de 1 p. 8 millions.

En résumé, des fragments de tissus de Cobaye furent placés pendant des périodes variant de 1 heure et demie à 24 heures dans de la solution de Tyrode contenant du virus filtré de stomatite vésiculaire. Ensuite, on les cultiva dans des flacons pendant 7 à 10 jours. Au bout de ce temps, les cultures furent retirées des flacons et inoculées à des Cobayes. Dans deux expériences, elles ne contenaient plus de virus. Ce fait

doit probablement être attribué à ce que les conditions très rigoureuses qui sont nécessaires à la conservation du virus en dehors de l'organisme (1) n'avaient pas été réalisées. Dans les trois autres expériences, le virus non seulement était resté actif, mais s'était considérablement multiplié. Son activité avait augmenté de 10 à 1.000 fois. Il est donc certain que, dans les tissus du Cobaye cultivés avec une technique convenable à une température de 39°, le virus vit et se multiplie. Ce résultat est frappant parce que, dans les conditions ordinaires, le virus périt rapidement à la température de 37°.

(1) P.-K. Olitsky. *Journ. of exper. Med.*, 1927, t. XLV, p. 969.

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EFFECT OF FORMALIN ON THE VIRUS OF VESICULAR STOMATITIS OF HORSES.

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(Received for publication, March 20, 1928.)

From time to time reports have appeared dealing with active immunization with filtrable or allied viruses that have been killed by weak solutions of formalin.

Among those recently making favorable reports are Cumming,[1] Vallée, Carré and Rinjard,[2] the British Foot-and-Mouth Disease Research Committee,[3] and Hunt and Falk.[4] Cumming worked with rabies virus treated less than 24 hours with 0.4 per cent formaldehyde, while Vallée and his coworkers studied foot-and-mouth disease virus exposed to 0.5 per cent formalin at 20°C. for from 4 to 7 days. The British Committee used the formalin in 0.1 per cent strength, which was added to filtered contents of foot-and-mouth disease vesicles, the mixture being kept for 48 hours at 26°C. before use. Hunt and Falk tested vaccinia material to which was added formaldehyde in 0.1 per cent concentration for 12 hours, the mixture being kept at unstated temperature. Besides these favorable reports, failures have been recorded. Thus Abramson and Gerber[5] were unable to secure immunization with formalinized virus of poliomyelitis.

It is obvious that the subject is one of wide interest and importance, for if active immunization can be secured with filtrable viruses, altered by this simple chemical means so that they are incapable of inducing specific lesions, a great step forward will have been taken. Up to the present, active immunity has been secured only by the use of attenuated or modified, but still living viruses, capable of producing some, although at times very slight, specific changes in the tissues.

[1] Cumming, J. G., *J. Infect. Dis.*, 1914, xiv, 33.

[2] Vallée, H., Carré, H., and Rinjard, P., *Rec. méd. vét.*, 1925, ci, 297; *Rev. gén. méd. vét.*, 1926, xxxv, 129.

[3] Second Progress Report of the Foot-and-Mouth Disease Research Committee, Ministry of Agriculture and Fisheries, London, 1927, 1–117.

[4] Hunt, L. W., and Falk, I. S., *J. Immunol.*, 1927, xiv, 347.

[5] Abramson, H. L., and Gerber, H., *J. Immunol.*, 1918, iii, 435.

Our study was made with a strain of the virus of vesicular stomatitis of horses, already described,[6,7] which has been shown to be closely related to the virus of foot-and-mouth disease. It was designed to determine (*a*) whether protection could be obtained with formalinized virus, and (*b*) whether the protection could be construed as arising from the effects of a killed antigen.

Before proceeding with the description of experiments, we wish to recall some earlier conclusions derived from tests with antiseptics on the virus of foot-and-mouth disease.[8] The remarkable resistance of the latter virus to certain chemicals was found to be due to the fact that the reagents coagulate the proteins of the medium in which the virus is ordinarily distributed. As a result of this coagulation, the positive charge of the virus, and its minute size, the active material is held within large coagula and prevented from coming into immediate contact with the chemicals. Similar conditions have been found to occur in the instance of the virus of vesicular stomatitis,[7] in which the virus proved resistant to many chemicals which coagulate proteins. Furthermore, one of us (Long) has found that still another chemical, namely, neutral acriflavine in 1 per cent solution, acts as a coagulant so that the virus proves resistant when submitted to it for at least 24 hours. Since formalin is a substance which coagulates proteins, we studied the virucidal action by means of a series of controls, as shown in the following tests.

Viability of Formalinized Virus.

In the preparation of formalinized virus of vesicular stomatitis, we followed mainly the methods employed by the British Foot-and-Mouth Disease Research Committee.[3] The Committee have studied the problem in connection with the virus of foot-and-mouth disease more extensively than any other recent workers.

[6] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx, 147.

[7] Olitsky, P. K., *J. Exp. Med.*, 1927, xlv, 969.

[8] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815; and the Report of the American Commission to Study Foot-and-Mouth Disease, to be published by the U. S. Bureau of Animal Industry.

Preparation.—The virus consisted of the clear fluid derived from vesicles in the pads of from three to over ten guinea pigs injected 24 hours previously, as a rule, with guinea pig passage virus of vesicular stomatitis. The fluid was aspirated into a syringe and diluted 1 in 50 with phosphate buffer solution at a pH of 7.5 or 7.6. The diluted material was filtered through a Berkefeld V candle and the major portion of the filtrate was used for formalinization and the remainder for control tests of pathogenicity in guinea pigs. Six lots of filtered virus were prepared at different times. To four, 0.1 per cent neutral formalin was added, to one, 0.5 per cent, and to the sixth, 10 per cent. The formalinized virus was kept in an incubator at 26°C. for 48 hours or longer, and then tested by injection into the pads of guinea pigs.

The 0.5 and 10 per cent formalinized material proved too injurious for use: extensive necrosis (or "wet" gangrene) of the pad followed the use of the 10 per cent mixture, and a somewhat less degree of destruction, the 0.5 per cent mixture. Although the virus was killed in both instances after 2 days at 26°C., the factors of injury, confusion of specific and non-specific effects, and mortality led us to abandon the higher concentrations of formalin in favor of 0.1 per cent of this chemical, which is the concentration generally employed in preparing the so called "vaccines" of filtrable viruses. As will be shown later, even 0.1 per cent formalin is not wholly free from injurious effects—a mild edema being sometimes induced in guinea pigs. Similar effects in rabbits were observed by Hunt and Falk.[4]

Titration of Filtered Virus.—In order to determine the activity of the original filtered virus before utilizing mixtures of it with formalin for immunization tests, it was essential to show that the specimens of virus employed produced the characteristic lesion in guinea pig pads. Of three samples accurately titrated, one induced experimental vesicular stomatitis in a 1:1,000,000 dilution, another in 1:500,000, and a third in 1:100,000 dilution. The remaining samples were injected, as a routine procedure, in dilutions of 1:100 or 1:1,000, all being capable at these dilutions of producing typical lesions.

From now on we shall designate as "vaccine" the 0.1 per cent formalinized virus. We wish again to emphasize the fact already mentioned that this strength of formalin has in some animals an injurious action on the pad tissues, as shown by edema following injection. This injurious action is uninfluenced by either strong or weak virus; hence it must be ascribed to the chemical alone.

Living Virus in the Vaccine.

Tests were first made for evidence of living virus in the mixtures with formalin. After the freshly made mixtures had stood 48 hours at 26°C., 0.5 cc. was injected into each posterior pad of at least two guinea pigs. The results were as follows:

Vaccine 2.—Two guinea pigs, A and B, injected. After 4 days, A showed a single vesicle on one pad, yielding clear, serous fluid. B exhibited a similar condi-

tion on the 3rd day. Guinea Pig B was etherized, the affected pad removed and ground in buffer solution with sterile sand. The pad emulsion was injected into the pads of two normal animals. Both developed typical vesicular lesions on the following day.

Vaccine 5.—Two guinea pigs, A and B, injected. On the next day, A showed a single vesicle on the right pad, while B was unaffected. 14 days after the original inoculation, B was injected in both pads with active virus. The following day, typical vesicles had appeared on both pads.

Vaccine 6.—Four guinea pigs, A, B, C, and *D*, injected. 24 to 48 hours later, all showed either single vesicles or no visible lesions; yet on pricking the pad with a needle, clear, serous fluid exuded. This clear fluid from Guinea Pig D was inoculated into the pads of Guinea Pig E, in which typical vesicles arose within 24 hours. Guinea Pigs A, B, and C were reinjected with active virus after 14 days, and as none responded they can be regarded as immune, a finding which confirmed our opinion that the initial lesions following the injection of vaccine were those of vesicular stomatitis.

Vaccine 7.—Three guinea pigs, A, B, and C, injected. After 24 to 48 hours, no visible lesions appeared, except edema. On pricking the pads with a needle, a clear, serous fluid exuded. Guinea Pig A was etherized, the pads removed, ground in buffer solution with sand, and the emulsion inoculated into the pads of a normal animal. The latter developed typical vesicles by the next day.

From the above four tests, it may be concluded that the so called vaccines as prepared with 0.1 per cent formalin contain living virus. In contrast, we wish to record that guinea pigs in which pad inoculation was made with 0.5 per cent and 10 per cent formalinized virus developed severe necrotic lesions, from the fluid contents of which the virus of vesicular stomatitis could not be recovered. One may infer that the virus is killed by these concentrations.

The experiments described indicate that the usual concentration of formalin (0.1 per cent) employed for the production of so called vaccines of filtrable viruses does not kill the virus of vesicular stomatitis after a contact of at least 2 days at 26°C. The fact should, however, be noted that the lesions induced by the treated virus are generally slight, and that they may be confused with edema resulting from the action of the formalin alone. Simple inspection of the inoculated site is not sufficient to determine whether the effect is wholly chemical or due in part to living virus. In order to determine this, one should prick the superficial skin with hypodermic needles so as to cause any existing fluid to exude, which fluid, or that obtained from the emulsi-

fied pad, should be reinoculated into normal animals and the effects observed. Finally, recovered guinea pigs should be subjected to a later injection of active virus to determine if immunity exists,[9] and any pads showing suspicious lesions should be examined histologically for the presence of characteristic pathological changes and of the inclusion bodies which we have already described.[10] Such has been our own routine procedure.

Since, as the tests described above show, 0.1 per cent formalin fails to kill the vesicular stomatitis virus after 48 hours contact, we next studied the point at which the infectiousness of the so called vaccine ceased.

Vaccine 7 (0.1 Per Cent Formalin.)—Three guinea pigs injected with this vaccine kept for 48 hours at 26°C. after its preparation, were found to harbor the virus, a fact demonstrated by the preceding protocol. Two of three animals which received the same material after it had stood for 72 hours at 26°C. also gave evidence of containing living virus, after transferring the ground pads of the two animals to normal guinea pigs, which, in turn, developed characteristic vesicles. Six guinea pigs injected with the preparation after it had stood for 96 and 120 hours at 26°C. exhibited no effects which could be attributed to living virus. These unaffected animals were reinoculated in the pads about 10 days later with active, undiluted virus. All responded promptly with characteristic vesicles.

It is plain that Vaccine 7 contained living virus for 72 hours but not for 96 hours. The fact should be noted, in addition, that a single injection into the pads of formalinized virus, unless living virus is present in it, fails to immunize the animals.

Effect of Repeated Injections of Killed Formalinized Virus.

In view of the fact that previous workers have reported the production of active immunity by means of repeated injections of formalinized vaccines in which they supposed the virus to have been killed, we studied the effect of repeated inoculations with some of our formalin-

[9] It has already been established[6,7] that in experimental vesicular stomatitis, the induction of vesicles, no matter how small or few in number, is followed by a solid resistance to undiluted active virus. The same conditions prevail in experimental foot-and-mouth disease of the guinea pig (Olitsky, P. K., Schoening, H. W., and Traum, J., *North American Vet.*, 1927, viii, 42).

[10] Olitsky, P. K., and Long, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 287.

ized virus. But, as the following protocol shows, we were unable to detect any immunity in animals "vaccinated" with dead virus.

Two vaccines were selected, Nos. 2 and 3, made with 0.1 per cent formalin, which had been shown to contain living virus after 48 hours at 26°C., and which had been kept thereafter in the cold (5°C.). After 34 and 39 days respectively at this temperature, the vaccines were injected into the pads of guinea pigs. No effects of living virus could be detected by the methods already described. None of the six animals of this series proved resistant to a later injection of active virus.

The same preparations were tested again after they had been kept at 5°C. for a longer period (70 days in the case of Vaccine 2 and 75 days[11] in that of No. 3). Twelve guinea pigs were given a series of seven injections with one or the other vaccine, six receiving 0.5 cc. intramuscularly and six others 1 cc. intradermally and subcutaneously, that is, 0.5 cc. in each of the posterior pads. The first six injections were given at 3 day intervals, and the seventh 4 days after the sixth. 3 days after the injections had been completed, the ten surviving animals were inoculated into the pads with virus of known activity. All responded within 24 hours with characteristic vesicles.

In the following experiment it will be shown that it is possible to induce resistance to active, undiluted virus by a single intramuscular or intracutaneous injection of living, untreated virus, although under these conditions no demonstrable lesions occur.

Fluid from vesicles 24 hours old of guinea pigs inoculated with passage virus, whether diluted 1:10 or undiluted, induced no demonstrable lesions when injected intracutaneously into the shaved skin of the abdomen, or intramuscularly into the thigh. In four of the seven animals thus treated, the pads were scratched, though not inoculated, at the time of the virus injection. These pads developed no lesions.[12] All the guinea pigs resisted pad injection of undiluted, active virus from 10 to 23 days later.

The virus of vesicular stomatitis, therefore, appears to behave as do some other filtrable viruses, that is, immunity can be induced only when living virus is employed.

[11] The experimenters of the British Committee state that the activity of their killed foot-and-mouth disease vaccines persists in the cold for at least 197days, and that protection is first noted 48 hours after injection.

[12] Absence of localization in the injured pad may be ascribed in these instances to the fact that the strain of virus which we used had lost its original feeble power to induce secondary lesions. The failure of the vesicular stomatitis virus to induce demonstrable lesions after intramuscular or intracutaneous injection was first noted in cattle.[6]

SUMMARY AND CONCLUSIONS.

The virus of vesicular stomatitis is not readily killed by formalin. This chemical is one of a group which coagulates the proteins of the medium in which the virus is usually contained. It has already been found[7] that other reagents of the protein-coagulating group are not actively virucidal, and the effect of formalin in this instance is therefore characteristic of the group.

The so called formalinized vaccines which give rise to immunity can be shown to have done so because of the presence of living virus. A single injection of such so called "vaccine," or of other material containing living virus, is capable of inducing immunity in guinea pigs. No protection, however, follows a single injection of dead virus. Furthermore, repeated inoculations of virus killed by formalin likewise fail to induce resistance against subsequent injections of the living virus.

It is concluded, with respect to the virus of vesicular stomatitis, that the use of formalin has failed to solve the problem of active immunization with dead virus.

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CYTOLOGICAL STUDIES ON MALARIA.

I. THE FLAGELLATION OF PLASMODIUM KOCHI AS OBSERVED WITH DARK FIELD ILLUMINATION.*

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When a small amount of fresh blood from a case of malaria is mounted on a slide under a coverglass without the addition of any foreign fluid the male gametocytes often undergo a remarkable series of changes. Very motile "flagella" appear at their surface, break away and swim about actively among the blood corpuscles in the plasma. This process of "flagellation" was first observed by Laveran (1) who at once stated his belief that the flagella represent a definite and important stage in the life cycle of the parasites. A few years later MacCallum (2) confirmed this view by demonstrating that the flagella in bird malaria are in fact true microgametes capable of fertilizing the female elements.

The presence of flagellating malarial parasites in the monkeys of the Pasteur Institute of Tunis and the observation by one of us (C.W.A.) that the process can be seen with great clearness by the dark field microscope were instrumental in leading the Director of the Institute, Dr. Charles Nicolle, to suggest that we make a detailed cytological study of flagellation by this means.[1]

* Contribution No. 1 of the North African Expedition of The Rockefeller Institute for Medical Research.

[1] A film was issued by Pathé Frères entitled "The blood of a bird (Padda) infected by a Hematozoon." While it does not show the details of the process it does indicate that valuable results may be secured by cinematographic study.

The information secured is obviously of a rather different character from that hitherto reported. It relates chiefly to changes in the viscosity of the parasite (measured by the Brownian and translationary movement of intracellular granules), to alterations in size and shape caused by endosmosis and exosmosis and to the mobility of the microgametocytes and microgametes. The study of fixed and stained smears gives little if any data regarding the time occupied by the various phases of flagellation, and the sequence of events can only be observed indistinctly and with difficulty by direct illumination; but, with the dark field microscope, and a stop watch, the time relations may be accurately determined. Nevertheless, dark field examination alone gives an even more incomplete and one sided picture of structural changes than does the study of fixed and stained smears supplemented by the examination of living parasites by direct illumination. For example, no traces are seen in the dark field of chemical alterations in the parasites, which do not lead to the development of light-reflecting surfaces. Areas of the cytoplasm rich in nuclear material often look exactly the same as regions almost wholly devoid of it. For these and kindred reasons, we decided to reinforce our dark field studies by using the polariscope, supravital dyes and various methods of fixation and subsequent coloration of the parasites.

I. Material.

The monkeys belonged to the genus *Callithrix* (very probably *C. personata*). The malarial parasite is *Plasmodium Kochi*. A chronic infection was first noted in a monkey imported from Senegal. A similar infection was produced in a second monkey by the inoculation of blood from the first animal. A third monkey, likewise from Senegal, was found to be naturally infected and the parasite was transmitted to a fourth in the same way. Three of the monkeys recovered naturally and one was killed in order to obtain tissues for examination.

In smears colored by Giemsa's stain, the male and female gametocytes are readily distinguished by the same criteria used in the case of human malarial parasites. The former (Figs. 2 and 3) are recognized by their large faintly colored nuclei and by the pale rose color of their cytoplasm and the latter (Figs. 1 and 2) by their small deeply staining nuclei and bluish cytoplasm.

Under dark field examination, the identification is more difficult because the nuclei are often invisible and the tinctorial reactions of the cytoplasm are obviously lacking. Nevertheless close observation shows that the male gametocyte is usually, but not always, slightly smaller and does not possess, near its margin,

a clear area free from illuminated granules like that occupied in the female gametocyte by the nucleus. In the male gametocyte, also, the granules seem to shine with a whiter, more ghostly hue than those in the female gametocyte.

II. Flagellation.

Flagellation was studied at room temperature in small amounts of fresh blood mounted on slides under cover glasses without the addition of any foreign fluids. The cover glasses were ringed with paraffin to prevent evaporation when it was desired to observe continuously over a fairly long period of time. With the dark field the parasites can be identified by the brilliant illumination of the granules within them, which are in intense Brownian movement, though the leucocytic granules in neighboring cells remain stationary.

The granules are certainly for the most part pigment, as may be seen by shifting the "Wechselkondensor" so that the same particles are viewed by direct rays of light; but it cannot be said that they are all pigment. Polariscopic examination shows that most of the pigment is not doubly refractile although a little of it appears to be. The doubly refractile pigment is concentrated at the periphery of the parasite. von Berenberg-Gossler[2] thought that none of it was doubly refractile. Supravital staining with Janus green, and the coloration with anilin fuchsin of smears fixed in osmic acid does not reveal the existence of any mitochondria among the granules of pigment. This is an observation so unexpected that the same means were employed in an attempt to bring to light the mitochondria in an allied parasite, *Hemoproteus columbæ*, likewise without success. Brilliant cresyl blue and nile blue sulphate, when employed as supravital stains, color a material which exists in the ground substance between the granules of pigment. In its reactions this material resembles the substance rendered visible in reticulated erythrocytes by the same treatment. The oxydase reaction is negative when applied to the gametocytes of both sexes and the male gametes, but a few granules may be colored blue in the young ring forms of the parasite. Neighboring polymorphonuclear leucocytes are strongly positive.

Plate 1 illustrates mature female and male elements and the process of flagellation, as seen in smears; and Plates 2, 3 and 4, the phenome-

[2] von Berenberg-Gossler, H., *Arch. f. Protistenk.*, 1909, xvi, 245.

non as viewed in the dark field. In the latter, the granules which are in active Brownian movement are represented in red, while those which are stationary are depicted in black. The size relations are indicated by the scales in microns.

1. Latent Period (30 seconds to 6 minutes). When the blood is examined immediately after mounting it is often impossible to detect with the dark field any sign of flagellation. But the process may begin almost instantaneously or be delayed for a few minutes. Neither are flagellating gametocytes visible in smears of absolutely fresh blood, although their occurrence under such conditions has been described. For instance, Lawson (3) writes, in reference to the examination of presumably fresh blood, that "Sexual flagellating parasites are only occasionally found in stained smears from the circulating blood but now and then a few examples may be seen."

2. Cessation of Brownian Movement (about 10 seconds). The first noticeable stage in the formation of the male gametes is the suppression of Brownian movement. Once it has commenced this is effected with great rapidity and is completed in approximately 10 seconds, measured with a stop-watch. The retardation and cessation of Brownian movement may take place simultaneously in all parts of the parasite but in fully 80 per cent of flagellating parasites it begins in the peripheral cytoplasm and spreads toward the center where the last moving granules are seen. (Figs. 16, 32, 48, 70, and 78.)

This sudden increase in viscosity, which amounts to a true process of gelation, is not accompanied by any detectable alteration in the volume of the parasite. In other words, the taking up or giving off of fluids, if it occurs, must be very slight. Very curious is the fact that when a small amount of slightly hypertonic sodium chloride solution is added to the blood under observation, the gametocytes will swell while the erythrocytes become crenated.

There are certainly marked alterations in permeability about the same time. The penetration of brilliant cresyl blue, neutral red, and other dyes of the same general category is restricted. The male gametocyte during this phase and all the subsequent stages of flagellation is much more difficult to stain supravitally than it was originally. Parasites colored supravitally before flagellation never even undergo this preliminary stage of gelation. Although it is of great importance,

we have not been able to ascertain which is primary, the alteration in viscosity or the change in permeability. Our impression is that they are coincident.

Nothing is really known of the nature of the exciting stimulus or stimuli which initiate these alterations in gametocytes about to flagellate except that they occur with an altered environment. Thomson (4) believes the stimulus to be osmotic in nature. He finds that flagellation is prevented, in the case of *Plasmodium falciparum*, by the addition of hypertonic salt solution, and in common with other investigators reports more rapid flagellation when a little water is added. Thomson mentions the deposition of lime salts during coagulation which is interesting in view of the preliminary gelation which we describe, although, in our preparations, flagellation precedes coagulation; indeed, coagulation may be long deferred. The process is seen apparently with equal clearness in fresh living blood mounted on a clean slide and in the gut of a mosquito. We find that it occurs when the slides and covers are warmed and the whole preparation is held at about body temperature and are able to confirm Ross's (5) observation that the flagella appear when the slides are kept on ice. Evidently temperature is not a controlling factor.

It is probably at this time that the microgametes (flagella) commence to be differentiated within the parasite. The earliest phases of this development are not visible with the aid of our dark field equipment and resort was made to methods of fixation and staining. Good results were secured: (1) by removing the cover glass to remain 30 seconds after the blood had been mounted, and then removing them at intervals of 10 seconds over periods of 20–30 minutes; (2) by rapidly smearing and drying the blood, (3) by fixation in methyl alcohol, and (4) by coloration with Giemsa's stain.

To discover just what degree of development of microgametes viewed in such preparations corresponds with the beginning of gelation is difficult. But it seems clear that very little architectural specialization could exist while the granules are in such active Brownian movement and also shift about from place to place as they do before gelation. There is every reason to believe that within the 10 seconds, mentioned above, the microgametes are built up in the cytoplasm. The process may be arbitrarily divided into three stages.

The first consists of a change in staining reaction of the parasite, and is recognized by Brug.[3] It comes to be colored much more readily by the red component of the stain (compare Fig. 5 with Fig. 4). This is due to an alteration in the nucleus with the discharge of chromatin-like substances into the cytoplasm. If a swing in the reaction of the cytoplasm toward the acid side should occur at this time it might conceivably initiate or facilitate the gelation. All our attempts to secure positive evidence regarding such a change were unsuccessful. Special indicators, such as azolitmin and erythrolitmin,[4] failed to penetrate the cells in sufficient concentration, and we were handicapped in our experiments by the shortness of available time in the latent period.

The second stage, likewise described by Brug, is characterized by the laying down in the peripheral cytoplasm of concentric strands of material parallel to the surface of the cell. This is illustrated in Fig. 5. They are not mentioned by von Berenberg-Gossler[2] although, like us, he examined *Plasmodium Kochi*.

At the third stage which may be regarded as beginning at this point our observations differ considerably from those of Brug. He describes the development of a chromatin network from the strands and at the nodal points of this network the differentiation of chromatin granules destined to form the nuclei of the microgametes. We see no signs of a network and in this connection our Figs. 6 and 7 should be compared with his Figs. 43–46. In our preparations, the strands of material simply become narrower and more concentrated and at the same time tend to be colored more intensely by Giemsa's stain. Another important alteration is the development within the bands of material, seldom at their extremities, of thickened areas which take the stain still more energetically and which we regard as the developing nuclei of the microgametes. Until now, the parasites retain their approximately spherical outlines.

It is to be remembered that Brug was dealing with a different parasite, *Proteosoma præcox*, which fact may explain the discrepancies between his account and ours although in Neumann's[5] figures of

[3] Brug, S. L., *Arch. f. Schiffs.-u. Tropen.-Hyg.*, 1916, xx, 289.

[4] Kindly prepared and given to us by Dr. P. D. McMaster of the Rockefeller Institute.

[5] Neumann, R. O., *Arch. f. Protistenk.*, 1909, xiii, 23–69.

Proteosoma præcox the network formation is absent. There is also the possibility that Brug's methods of fixation created an artificial network. He did not control them by observations on the living. In the case of *Plasmodium vivax*, Schaudinn[6] describes quite a different process of flagella formation. The nuclear substance breaks up into "chromatin islands" one of which goes to each microgamete and is usually placed approximately equidistant between its extremities. The laying down of concentric strands is not mentioned.

In a female, or macrogametocyte, subjected to the same environmental influences, there is no gelation and the granules continue to exhibit active Brownian movement. The sequence of changes which they undergo is illustrated for purposes of comparison in Figs. 39–46.

How general is this process of gelation as a stage in microgamete formation we cannot say. In the case of *Hemoproteus columbæ*, which we have studied in the same way, the viscosity changes are curiously reversed. Before flagellation the pigment granules, which are all doubly refractile, are stationary and show no signs whatever of Brownian movement. Brownian movement does not, indeed, set in until the microgametes begin to extend. It is true that the majority of the granules in the male gametocyte are larger than those in *Plasmodium Kochi* and hence would be less likely to exhibit motility of this kind, but there are some very fine ones which remain immobile although one would expect them to respond immediately to lowered viscosity by characteristic movements. Flagellation as observed principally in fixed preparations of *Hemoproteus columbæ* is described by von Wasielewski and Wülker (6).

3. Elbowing Movements (duration about 30 seconds). In rare cases, the process of gelation may not be followed by elbowing movements. When this happens no microgametes are formed (Figs. 69–76). Generally, however, under the dark field the whole gametocyte is seen to quiver. Blunt processes are extended, as if due to some pressure from within, and retracted several times. During these movements the gametocyte loses its spherical shape and becomes bluntly angular, changing in form from moment to moment (see Figs. 17, 33, 49, and 78).

[6] Schaudinn, F., *Arb. K. Gesundheitsamte*, 1902, xix, 169.

What seemed to be the other process was also directly observed in the living condition but it does not result in the liberation of a microgamete.

Reference may here be made to the observation recorded in Figs. 99–119. A male gametocyte possessed of a large motile extension was observed (Fig. 99) at 2.50 p.m. Four minutes later this became bifurcated at the end (Fig. 100). This bifurcation disappeared and the distal half shrank (Fig. 101). Then the extension curved around the parasite (Fig. 102), became free again, curved around again and was finally incorporated in the substance of the parasite about 3.03 p.m. At 3.12 a process bellied out from the parasite (Figs. 108, 109). This became free at one end and exhibited typical motility (Fig 110), curved back finally (Fig. 111) and was absorbed (Fig. 112).

During extension of the microgametes certain changes, rather variable in character, take place in the parasite. The pigment granules may become condensed into a very dense mass (Figs. 18, 34, and 50), the remainder of the cytoplasm being optically homogeneous with the equipment employed except for a few extremely minute particles in very active Brownian movement (illustrated in red) and for one or more large clear vacuoles (Figs. 22–26). The minute particles are not visibly pigmented when the condenser is shifted so that they are viewed by direct rays of light. They do not take up Janus green, nor are they doubly refractile when viewed with crossed Nicols.

In other cases, however, the microgametes are extended without the development in the cytoplasm of the gametocyte of such relatively clear areas (Figs. 78 and 85).

The site of extension is variable. They may be pushed out from several points in the circumference of the gametocyte or from only one side of it (Fig. 35). In the latter case, their combined movement drags the gametocyte about rapidly among the corpuscles. The extension of all the microgametes may occur almost simultaneously, that is to say over an interval of approximately 5 seconds, or they may be shot out successively one after another and thus consume a variable length of time up to nearly 5 minutes.

The structure of the attached microgametes is not very uniform. Some are much thicker than others. Compare for instance the seven microgametes attached to the cell represented in Fig. 35. Their

average girth is noticeably greater in Fig. 85 than in Fig. 80. The process illustrated in Figs. 99–102 is unusually thick. The thin ones appear in the dark field to consist only of a very delicate brightly shining filament, while, in the case of the more robust microgametes, two illuminated contours may be distinguished enclosing a core which is optically homogeneous with the equipment employed except for the presence of one or more granules. These granules are of at least two kinds.

Some are merely droplets of pigment discharged from the microgametocyte but such are of rare occurrence and are only found in the thickest microgametes. These thick microgametes seem to differ only from the thinner ones by the possession of a larger contribution of cytoplasmic material from the microgametocyte. Sometimes they carry fairly large localized swellings (Figs. 8, 9, and 14). By virtue of this more fluid constituent the pigment is not necessarily stationary but occasionally moves back and forth along the attached microgamete—a phenomenon already noted by Laveran.

In other instances it seems certain that the brightly shining granule is in fact the nucleus of the microgamete. This nucleus may occur more or less equidistant between the two extremities but is more commonly observed in what we may call the "head end." The "head end" is distinguished from the "tail end" when viewed in the dark field not only by the presence of this shining granule but also by the fact that it is bluntly rounded, not pointed (Figs. 85–87). The "head end" is the extremity last to become separated from the gametocyte. This may be seen in stained smears by reference to Figs. 11 and 12. Occasionally the nucleus may consist of two rod-shaped particles arranged end to end as represented in Fig. 9. But microgametes are occasionally met with in smears as well as under dark field observation, in which no such nucleus-like structure can be made out.

While the microgametes are still attached they exhibit two distinct types of motility: First a quick *serpentine* movement in which the body of the gamete is thrown into two or more curves. This is illustrated in Fig. 76. The "head end," because it is still attached to the gametocyte, cannot move from side to side with the amplitude represented by the length of the dotted line, so that the motion is communicated to the tail end which lashes about from side to side instead of

merely following after the "head end." Progression would take place in the direction of the arrow were it not prevented in this way by the gametocyte. This may correspond to the "undulatory" type of movement reported by Manson.[7] He likens it to the movement of a spirillum and says that it serves for locomotion.

The second type of movement is even more rapid (see Fig. 68). If the gamete were free and not anchored to the microgametocyte, this movement would propel it in the opposite direction. In character, it is *revolutionary*, that is to say the whole microgamete, while itself only slightly curved, circles about a long axis in the position of the arrow in the diagram. It is a kind of boring motion. Perhaps this is the rapid *oscillatory* movement of Manson. He says that it is especially observed when the flagellum during its voyages meets a red blood cell or some other obstacle.

Both kinds of movement may be observed in neighboring microgametes attached to a single microgametocyte. In their contortions the microgametes brush up against one another but seldom become entangled. Their surfaces are not sticky.

Not all the extended and typically motile microgametes are discharged. In exceptional cases some pass back again into the gametocyte as already described (Figs. 21–25). A few lose their motility and either degenerate in the extended condition or are resorbed by the gametocyte.

A good example is afforded by the cell illustrated in Figs. 85–93. This cell discharged a number of normal and vigorous microgametes between 2.50 when the blood was mounted and 3.13 p.m. when Fig. 88 was drawn. After this time we may follow the fate of microgametes "a" "b" and "c." Microgamete "a" retained its motility longest (3.31 p.m.) but became progressively smaller and smaller in size (Fig. 93). Microgamete "b" expanded at its distal (tail end) where it became angular and attached to the slide (Fig. 89). Its motility was lost at 3.25 p.m., and, like "a," it became much reduced in size. Microgamete "c" became shrunken at the end (Fig. 88) and was finally (3.45 p.m.) completely resorbed by the gametocyte. In another case (Monkey 2), all of the six extended gametes were withdrawn and none were eventually discharged.

[7] Manson, P., Histoire de la vie des Germes de la malaria hors du corps humain, *Arch. de Méd. Expér.*, 1896, viii, 524.

No evidence was found that the microgametocyte extends any processes other than the microgamete, such as ordinary pseudopodia, or the "protoplasmic attaching process" described by Lawson (3) who, however, did not employ the dark field. All the processes observed exhibited at some stage the very unique motility of the microgametes. The act of extension is more rapid and wholly unlike that of the protrusion of pseudopodia.

5. The Separation and Discharge of Microgametes (2 seconds to 5 minutes). This stage in flagellation logically follows extension of the microgametes but one commonly finds that they overlap. After some microgametes are discharged others continue to be extended. The time consumed in separation is arbitrarily taken from the moment when the microgamete attains its maximum extension. Separation may take place almost instantaneously or be deferred for several minutes. The microgamete twists itself free by the revolutionary movement before mentioned and departs tail first. The separation may be a clear one or the microgamete may drag out after it an extension of adherent and highly viscid cytoplasm so that it darts about for 30 seconds or more like a fish on a line.

6. Behavior of the Liberated Microgametes. Motile microgametes can be followed about in the microscopic field for at least an hour and a half in sealed preparations. They exhibit the serpentine and revolutionary types of movement described above. By the former they move forwards, or head first, and by the latter, backwards. The movement backwards is approximately four times as rapid.

As long as the power of movement remains a definite oscillation between progress in a forward and in a backward direction is maintained. It is not a question of encountering some obstacle. While passing without hindrance head first the microgamete will suddenly reverse and go tail first and *vice versa*. On the whole the periods of backward or revolutionary movement are of somewhat longer duration than those of forward movement soon after liberation as compared with an hour later. There are intervals of rest which grow longer and longer. Such an interval may occur at the time of reversal or during passage forwards or backwards. The last movement of the microgamete may be of either kind.

The direction of progression is entirely haphazard and at random.

While the microgamete usually zigzags about in a restricted area of the preparation it may travel relatively large distances. It butts up against red blood cells and leucocytes indiscriminately. When displacement currents occur in the preparation the microgamete may pass with the stream, against it, or it may cross it at right angles or diagonally. Each microgamete pursues a solitary course. Two or more never move side by side, or clump together. Either extremity may momentarily become adherent to a red blood cell or to a leucocyte, but the body does not appear to be equally sticky.

The rate of movement is estimated by measuring with the stopwatch the time required for the microgamete to pass across a known field of microscopic vision of known diameter. By the most rapid or revolutionary type of movement they travel about 2/5 mm. per minute or, expressed in other terms, 24 mm. per hour. The slower serpentine motion propels them at about 10 mm. per hour. These figures only very roughly approximate the true speed because the microgametes seldom cross the field of the microscope in a straight line and almost invariably collide with erythrocytes and leucocytes, which necessitates the making of difficult corrections in the measurements.

The strength of movement is illustrated by the observation that a microgamete attached at one end to a microgametocyte, when lashing about, is capable of indenting a red blood cell to a distance equal to a third of its diameter and of repeating the process with each swimming movement. It is noteworthy that although the corpuscle is thus indented the microgamete is so rigid that at the point of contact its own diameter is not diminished. The microgametes are less vigorous and shorter lived in monkeys which are recovering from an infection than in those in which the infection is beginning or at its height.

The majority of microgametes ultimately degenerate in the plasma. Their ability to move may be lost though they still retain their length and diameter unmodified. Slowly their outlines fade, so that gradually they reflect less and less light until they can no longer be distinguished. During this form of degeneration, they frequently adhere to the slide or cover glass. Or, while still capable of both kinds of movement, they may gradually swell and come to look almost like a trypanosome. They superficially resemble oökinetes. These enlarged forms may continue to move about the preparation for 15 or 20 minutes when they

become adherent and like the others seem to fade away. Never in either type of degeneration are granules formed within the microgamete. Take a specific example: Figs. 61–66.

We reserve for subsequent investigation by dark field examination coupled with the more usual methods the act of fertilization of the macrogamete by the motile microgamete which can better be studied in the gut of the mosquito.

7. Degeneration of the Microgametocyte from Which the Microgametes Have Been Discharged. The behavior of the microgametocyte after flagellation is variable. It may continue to be spherical with no noticeable change for as long as 40 minutes when it proceeds to shrink, giving off fluid. It becomes angular. The granules within do not undergo Brownian movement but heap up in the peripheral cytoplasm where they coalesce. An inert shell-like mass remains (Fig. 93). On the other hand, the microgametocyte may take up fluid at one point in its circumference where a swelling forms. Into this swelling escape granules which enter upon Brownian movement (Figs. 27–29, 72, and 74). Finally the microgametocyte ruptures through this swelling and its contents pour out into the plasma (Figs. 118 and 119). A microgametocyte from which the microgametes have been discharged, as seen in stained smears, is illustrated in Fig. 13. The remains of the microgametocyte, after flagellation, are often phagocytized by the polymorphonuclear leucocytes.

DISCUSSION.

These dark field studies give more precise information as to the time consumed in the different stages of flagellation than the methods commonly employed, and they tend to emphasize the physical changes that occur. Two observations seem to be of interest:

First, there is always a preliminary gelation. The protoplasm of the microgametocyte before flagellation is of low viscosity as indicated by the very intense Brownian movement of the contained granules. Gelation sets in first at the periphery and spreads to the center of the parasite so that the granules become stationary. This gelation is not accompanied by a noticeable alteration in volume and suggests the desirability of experimental studies with calcium salts to determine the exciting factors in flagellation.

Secondly, after extension and discharge, an oscillation is observed between a serpentine forward movement and a revolutionary backward movement. It is very difficult to conceive of a mechanism which would make this possible. The probability is that some kind of internal organization exists within the microgametes which has not yet been discovered. Without such internal architecture, it would be impossible to comprehend the distinct polarity and the rigidity combined with elasticity of the microgametes. If their fluidity were unassociated with organization, they would tend to round up into spheres in terms of the law of least surfaces: They certainly could not maintain a length twenty-five or more times their diameter.

For comparison with *Plasmodium Kochi*, we have examined by dark field illumination as well as through stained smears the process of flagellation in a related parasite, *Hemoproteus columbæ*. In respect to the phenomenon of gelation, the stages passed through are very different. The pigment granules before flagellation are stationary, never, in our experience, exhibiting active Brownian movement, as is always the case with *Plasmodium Kochi*. During and after flagellation, the viscosity is lowered instead of being increased and the granules undergo Brownian movement and change in position. The viscosity alterations are, therefore, curiously reversed.

Wenyon[8] refers to the insufficiency of information available in the separation of the species of plasmodia in birds and the same holds for certain mammals, especially monkeys. Diagnostic attributes of a kind not hitherto emphasized may perhaps be secured by dark field examination, with a minimum of technical effort. They would, of course, not replace the determination of species on the basis of fixed and stained smears; they would merely supplement and reinforce it.

SUMMARY.

The following stages may be recognized in the formation of the microgametes of *Plasmodium Kochi* by the dark field examination and by methods of fixation and staining.

1. There is a *latent period* of from 30 seconds to about 6 minutes

[8] Wenyon, C. M., Protozoology, New York, William Wood, 1926, xi, p. 978.

after the blood has been mounted under a cover glass during which no visible alterations take place.

2. Then the Brownian movement of the contained granules is suppressed first in the peripheral cytoplasm and then throughout the gametocyte. This is the stage of *gelation* and is completed in approximately 10 seconds. Evidence is advanced from the study of smears that it is during this period that the microgametes are differentiated in the form of strands of material just beneath the cell membrane which become nucleated.

3. The parasite then quivers, blunt processes are slowly extended and withdrawn, in other words we have the phase of *elbowing movements* which lasts for about 30 seconds. It is likely that the movements are caused by the motility of the contained microgametes, which, during this brief interval, are becoming further differentiated and practically attain maturity.

4. The microgametes are extended with extraordinary rapidity, each in a fraction of a second either from a restricted portion of the parasite or from all sides. As many as ten may appear but not always simultaneously. This stage of extension is, in the majority of cases, completed in from 5 seconds to 10 minutes. The act of extension may be effected in two ways. The microgametes either dart out head or tail first or their central part bellies out from the microgametocyte and one end becomes detached so that they flap free. While still attached, they exhibit both serpentine and revolutionary types of movement.

5. The separation or *discharge* of microgametes normally consumes from 2 seconds to 5 minutes. This stage overlaps the preceding because some microgametes continue to be extended while others are being discharged. The microgametes twist themselves free by revolutionary movement and depart usually tail first. The separation is generally clear cut but the microgamete may drag out after it a delicate thread of viscid cytoplasm.

6. The liberated microgametes exhibit a curious alteration between the serpentine forward movement and the revolutionary backward movement entirely irrespective of any obstacles which they may encounter.

DESCRIPTION OF FIGURES.

The figures in Plate 1 were drawn at the table level with camera lucida, Zeiss apochromatic objective 2 mm. 1.40 aperture, and ocular 18 of blood smears dried in air, fixed in methyl alcohol and colored with Giemsa's stain.

The microgametocytes in Plates 2–4 were likewise drawn at the table level with a camera lucida; but the microgametes moved so continuously and actively that it was usually impossible clearly to outline them in this way so that they had to be sketched in free hand. For the drawings on these three plates Zeiss apochromatic objective 3 mm. X with iris diaphragm was used with ocular 18 giving a magnification of 1856 diameters. Indirect illumination was secured by Zeiss paraboloid Welchselkondensor and the parasites were alive in blood mounted simply under a cover glass.

PLATE 1.

FIGS. 1 and 2. Female gametocytes.

FIGS. 3 and 4. Male gametocytes.

FIG. 5. An early phase in flagellation occurring in the stage of "gelation" (p. 18) before the "elbowing movements" set in. Bands of material, the future microgametes, are being laid down in the cytoplasm.

FIG. 6. A little further advanced. The microgametes stain more intensely.

FIG. 7. Still more advanced. Two of the intracellular microgametes possess distinct rod-shaped nuclei.

FIG. 8. Probably toward the end of the stage of "elbowing movements." The microgametes are further differentiated.

FIG. 9. A parasite with one fully extended microgamete and another microgamete in the act of being flapped out (p. 23).

FIGS. 10 and 11. Parasites with extended microgametes.

FIG. 12. A parasite with a single microgamete remaining attached in which the destruction between the head-end containing the nucleus and the pointed tail-end is clearly seen.

FIG. 13. A degenerating microgametocyte after the discharge of the microgametes.

FIG. 14. Three free microgametes. Note the swelling in one and the nucleus in another.

PLATE 2.

April 17, 9.55 a.m., blood of Monkey 2 mounted 18–21°C.

FIG. 15. 10.02, microgametocyte showing active Brownian movement. It exhibits a slight hyaline bulging at one side which is in reality a residue of the erythrocyte in which the organism was parasitic.

FIG. 16. 10.06, the Brownian movement slows down first at the periphery and then also in the center.

FIG. 17. 10.06 ½ elbowing movements of the microgametocyte occur.

FIG. 18. 10.07 ½, some fluid is taken in, the pigment is clumped and a few small granules in intense Brownian movement become visible in the area not occupied by pigment.

FIG. 19. 10.08, three microgametes (a, b and c) are extended almost simultaneously.

FIG. 20. 10.09, three more (d, e and f) are extended.

FIG. 21. 10.10, two more (g and h) are extended and "b," "c," "e" and "f" are discharged.

FIG. 22. 10.11, microgametes "d" and "h" are discharged and "a" and "g" are slightly withdrawn into the microgametocyte which changes its shape as represented and which develops in its interior a vacuole-like space. The particles in Brownian movement are increased in number.

FIG. 23. 10.12, microgamete "g," which like the others is typically motile, is extended for some distance and retracted (see the double arrow).

FIG. 24. 10.14, microgamete "g" is again extended and retracted. The membrane of the microgametocyte bulges rapidly in and out many times at the two places indicated by the double arrows. There is a slight increase in the granules in Brownian movement.

FIG. 25. 10.18, microgamete "a" is completely retracted and microgamete "g" very nearly so. The bulging in and out of the membrane continues at the point on the left already illustrated. A small blunt hyaline extension appears and disappears again in 6 seconds. The granules in Brownian movement are arranged differently and seem fewer in number. What may be a ninth microgamete ("i") is shot out to the side and withdrawn along the dotted line in approximately 8 seconds; but we have the impression that this is microgamete "g" reappearing in a different place. The microgametocyte itself shrinks slightly.

FIG. 26. 10.19, the microgametocyte enlarges considerably, a second vacuole-like space appears within it and a very motile microgamete ("i" or "g") is discharged.

FIG. 27. 10.21, there is some shrinkage, the vacuoles disappear, the cytoplasm seems to be partly partitioned and the motile granules are divided into two clumps. A projection of cytoplasm persists at the point where microgamete "g" was extended and retracted which consists probably of the cytoplasmic investment which covered it.

FIG. 28. 10.25, a slight alteration in shape of the mass of pigment takes place.

FIG. 29. 10.30, the group of motile granules is lost. Observation discontinued.

April 18, 9.47 a.m., blood of Monkey No. 2. 19–20°C.

FIGS. 30 and 31. 9.50–9.51, a typical microgametocyte with granules in intense Brownian movement.

FIG. 32. 9.52, cessation of movement begins at periphery and spreads to center. There is no noticeable change in volume.

FIG. 33. 9.52½, elbowing movements occur.

FIG. 34. 9.52¾, elbowing movements stop, pigment is concentrated in a roughly spherical mass, fluid is taken in and minute dancing particles appear in the otherwise optically clear cytoplasm. A constriction begins in the cytoplasm.

FIG. 35. 9.53, the constriction deepens, the clear cytoplasm expands and 7 microgametes are extended.

FIG. 36. 9.54, the portion consisting of unpigmented cytoplasm enlarges still more, a vacuole appears in it and some of the dancing particles may be observed to be definitely filamentous in shape. The constriction is more noticeable. Five of the microgametes are discharged.

FIG. 37. 9.56, the microgametocyte now divides into two parts. The unpigmented portion takes a spherical shape. The remaining 2 microgametes are discharged from the pigmented part.

FIG. 38. 10.06, there is but little modification in the unpigmented portion. The pigment in the other part becomes marginated over about two-thirds of the circumference and the fluid contents of the resulting mass are lost. Observation discontinued.

April 18, 10.17½ a.m., blood of Monkey No. 2 mounted.

FIG. 39. 10.18, a female or macrogametocyte surrounded by the remains of the membranes of the parasitized erythrocyte. The pigment granules are in active Brownian movement.

FIG. 40. 10.21, slight change in shape.

FIG. 41. 10.44, further change in shape. Some of the pigment assumes the form of short rods and rings. The configuration of the pigment-free areas is altered.

FIG. 42. 10.49, some pigment escapes.

FIG. 43. 10.51, irregular cessation of Brownian movement begins and the outlines of an oval structure may be distinguished in the unpigmented area. The macrogametocyte is reduced in size.

PLATE 3.

FIG. 44. 10.59, gelation progresses.

FIG. 45. 11.00, a faint series of tiny opalescent rods and threads appears in the ground substance between the pigment granules.

FIG. 46. 11.05, Brownian movement almost stopped. The outlines of the pigmented mass become angular. Observation discontinued.

April 24, 9.32 a.m., blood of Monkey No. 2 mounted 18–19½°C.

FIG. 47. 9.40, microgametocyte with intense Brownian movement. Small area where granules are very dense. A few granules stationary (represented in black).

FIG. 48. 9.41, peripheral Brownian movement begins to stop. The granules are drawn together leaving a small clear area at the periphery.

FIG. 49. 9.42, Brownian movement almost ceased. The cell quivers. Active projection and retraction of pigmented cytoplasm in the direction of the double arrow. The pigment granules clump together, leaving a large area free except for two particles in intense Brownian movement which are not visible with direct illumination.

FIG. 50. 9.43, Brownian movement completely arrested in pigmented area. The movements of this area less active. The clear area now contains several

motile microgametes. Through their action the cell membrane undergoes a wave-like series of movements in the direction of the arrow. The whole cell rotates in the same direction three times. The microgametes seem to be entirely separate from the pigmented area.

FIG. 51. 9.44, slight swelling of microgametocyte. The pigmented area is more condensed and the movements in it have stopped. The intracellular microgametes are more numerous and more highly refractile. They are very motile. The wave-like movements of the cell membrane continue and the microgametocyte rotates once more in the same direction.

FIG. 52. 9.45, a slight equatorial constriction occurs. The movement of the microgametes is slowing down and there is no rotation.

FIG. 53. 9.50, pigmented area becomes more angular. The microgametes are no longer motile and their refractive index decreases.

FIG. 54. 9.53, there is further condensation of the pigment and continued decrease in refractive index of the microgametes so that it is more difficult to see them.

FIG. 55. 9.55, the cell enlarges, becomes more spherical.

FIG. 56. 10.02, the swelling continues slightly.

FIG. 57. 10.03, the microgametes are still more difficult to detect.

FIG. 58. 10.13, only small traces of the microgametes may be seen.

FIG. 59. 10.42, microgametes no longer visible. The cell membrane ruptures in three places.

FIG. 60. 10.58, cell membrane temporarily reconstituted. Observation discontinued.

April 25, 9.25 a.m., blood taken from Monkey No. 3 mounted. 9.27, gametocyte observed with slow movement of granules.

FIG. 61. 9.31, microgamete discharged. Active movement in both directions.

FIG. 62. 9.35, movement slower. Periods of a few seconds without movement. Tends to take an arc-like shape. Small swelling of one end.

FIG. 63. 9.35½, becomes fixed to the slide at the swollen end. The other extremity moves through an arc as indicated by the dotted line.

FIG. 64. 9.36½, movement ceases. Great shrinkage. Rapid fading of outlines.

FIG. 65. 9.36¾, further shrinkage and fading.

FIG. 66. 9.37, only a small irregular motionless mass remains.

FIG. 67. A diagram to illustrate the serpentine type of movement of the microgamete. The body is thrown into two folds. The head moves from side to side with about the amplitude indicated and the microgamete passes in the direction of the arrow.

FIG. 68. A diagram to illustrate the revolutionary type of movement of the microgamete. The body, only slightly curved, revolves very rapidly about the axis represented by the arrow and is propelled tail first.

April 26, 2.35 p.m., blood of Monkey No. 2 mounted and ringed with paraffin, 18.5°C.

FIG. 69. 3.41, male gametocyte. Brownian movement active.

FIG. 70. 3.42, Brownian movement stopping.

FIG. 71. 3.42½, Brownian movement arrested. Very slight trembling movement of gametocyte.

FIG. 72. 3.43, optically hyaline protrusion at one side and clumping of pigment at the other. Increase in size. Brownian movement of tiny granules in the clear area.

FIG. 73. 3.44, increase in size.

FIG. 74. 3.45, becomes attached to coverslip at pointed end. Current of blood passes it.

FIG. 75. 3.49, detached and drifted in blood stream. Retained pointed shape in spite of many contacts with corpuscles.

FIG. 76. 4.18, shape still maintained indicating high degree of rigidity.

April 26, 3.55 p.m., blood of Monkey No. 3 mounted and ringed with paraffin, 19–20°C.

FIG. 77. 3.56, male gamete. Brownian movement slowing down.

FIG. 78. 3.56½, Brownian movement stopped. Rapid projections and retractions of cell.

FIG. 79. 3.56¾, shrinkage. Two flagella formed and discharged. Were not seen to arise. Appear to flash into visibility fully formed.

FIG. 80. 3.57, further shrinkage. Six more flagella formed and discharged very rapidly.

FIG. 81. 3.51½, spherical mass of pigment.

FIG. 82. 4.01, hyaline projection formed.

FIG. 83. 4.02, increase in size and resumption of Brownian movement. Breaking away of one side of pigment mass.

FIG. 84. 4.05, further swelling and disintegration.

PLATE 4.

April 19, 2.50 p.m., blood of Monkey No. 1 mounted.

FIG. 85. 3.03, a microgametocyte possessed of six very motile microgametes number "a" to "f."

FIG. 86. 3.04, Microgametes "d," "e" and "f" are discharged and the microgametocyte rotates slightly in the direction of the arrow.

FIG. 87. 3.06, the microgametocyte rotates farther in the same direction and Microgamete "b" expands and loses its motility.

FIG. 88. 3.13, Microgamete "b" contracts a little. The distal two-thirds of "c" also contracts.

FIG. 89. 3.16, the distal extremity (or tail end) of "b" becomes expanded and attached to the slide at two points, the intermediate portion is slightly motile. The contracted part of "c" is withdrawn into the microgametocyte.

FIG. 90. 3.25, Microgamete "a" shortens and becomes swollen but is still motile.

FIG. 91. 3.31, all three microgametes shrink.

FIG. 92. 3.45, Microgamete "c" is completely resorbed by the microgametocyte.

FIG. 93. 4.05, further shrinkage in, and fading of the outlines of Microgametes "a" and "b." The pigment becomes marginated and the contours of the microgametocyte angular. Its fluid contents seem to have been expelled so that it persists as a kind of shell. Observation discontinued.

April 19, 2.34 p.m., blood of Monkey No. 2.

FIG. 94. 2.46, microgametocyte with 6 microgametes attached to a small area of its circumference.

FIG. 95. 2.47, Microgametes "a," "b" and "c" are discharged. At the point indicated by the double arrows the membrane of the microgametocyte is rapidly extended and retracted for a period of about 15 seconds. Then the entire microgametocyte becomes enclosed with a second membrane which is very indistinct at first and later reflects more light. Within the clear space thus enclosed a very small Microgamete "g" makes its appearance and lashes about as represented.

FIG. 96. 2.48, the pigmented mass rotates so that the point of attachment of Microgamete "g" shifts to the left.

FIG. 97. 2.50, Microgametes "d," "e" and "f" are discharged and "g" disappears by rapid fading of its outlines. The pigment is concentrated.

FIG. 98. 3.01, the pigment is dispersed again and a few particles show Brownian movement. Observation discontinued.

April 13, 2.38 p.m., blood of Monkey No. 1 mounted. 18–20½°C.

FIG. 99. 2.50, microgametocyte with a large microgamete which exhibits both serpentine and revolutionary types of movement, but chiefly the former. The microgamete possesses an optically homogeneous core which appears to be continuous with a clear portion of the cytoplasm. The membrane of the microgamete is in the same way continuous with that of the microgametocyte. It is impossible to say just where one ends and the other begins. The pigment is concentrated. There is Brownian movement of granules which are invisible by direct illumination.

FIG. 100. 2.54, the microgamete becomes formed.

FIG. 101. 2.58, the free extremity (tail end) shrinks and losing its core is visible only as a brilliantly shining filament. The cell rotates as indicated.

FIG. 102. 2.59, the rotation continues, the pigment changes position and the microgamete curls about the microgametocyte.

FIG. 103. 3.00, the microgamete is again extended, this time much thinner and without any core but similarly motile. A second microgamete is shot out.

FIG. 104. 3.02, the first microgamete becomes applied to the cell a second time and the second microgamete curls about the cell so that only a trace of it remains.

FIG. 105. 3.04, no microgametes are visible. The microgametocyte is slightly constricted equatorially.

FIG. 106. 3.05, the constriction almost disappears.

FIG. 107. 3.09, the microgametocyte becomes slightly more rounded.

FIG. 108. 3.12, a clear hyaline swelling appears on site of first microgamete.

FIG. 109. 3.13, it enlarges.

FIG. 110. 3.14, refractile line becomes detached at one end with formation of what is apparently a third microgamete but which may be in reality a reappearance of one of the other two. It is typically motile.

FIG. 111. 3.15, there is slight rotation of the parasite and this microgamete curves in again and becomes attached at what was its free end.

FIG. 112. 3.15½, the microgamete is completely absorbed.

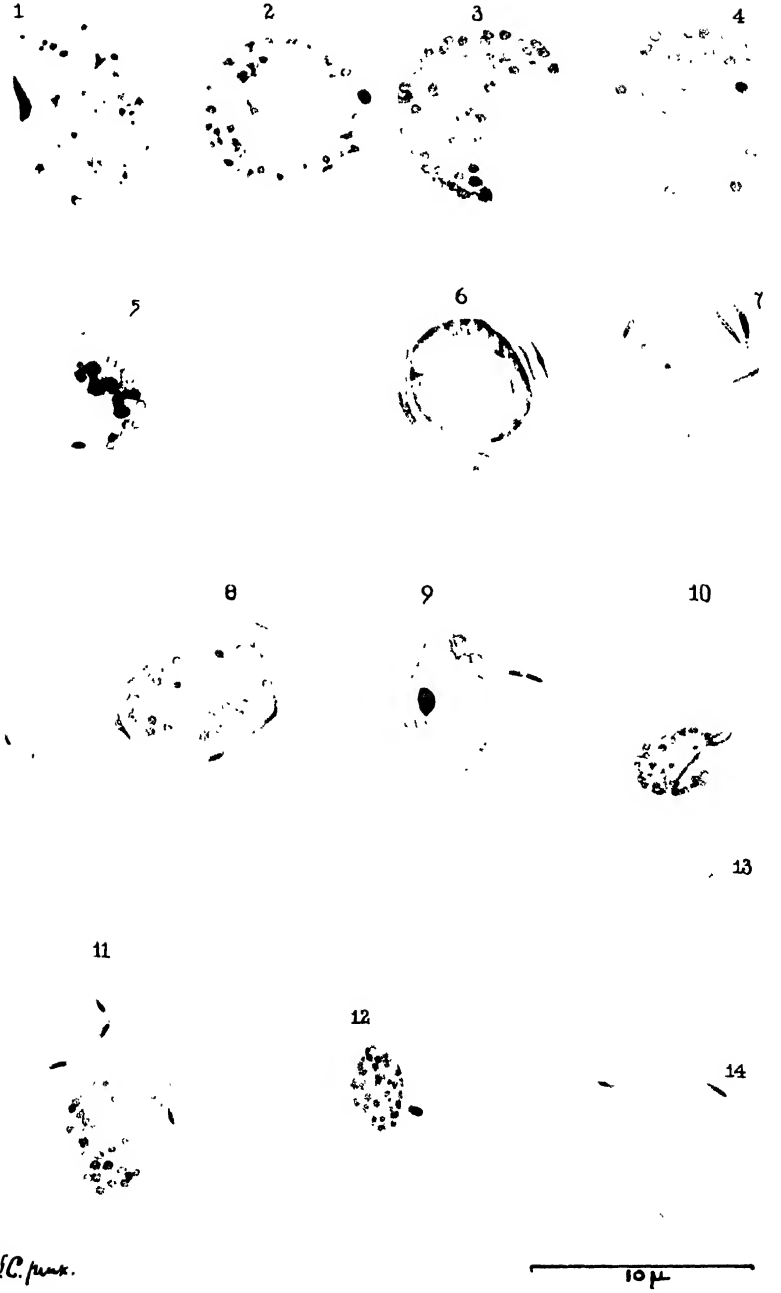
FIGS. 113 to 117. 3.21 to 4.05, the microgametocyte takes up water, the pigment assumes a central position and there is a considerable number of small particles in lively Brownian movement.

FIG. 118. 4.35, the microgametocyte ruptures.

FIG. 119. 4.51, the escaped contents round up to form two independent masses and the opening closes. Observation discontinued.

BIBLIOGRAPHY.

1. Laveran, A., *Rev. Scientifique*, 1882, xxix, 527.
2. MacCallum, G. A., *Lancet*, 1897, xi, 1240.
3. Lawson, M. R., *J. Exper. Med.*, 1914, xx, 526.
4. Thomson, David, *Ann. Trop. Med. and Parasit.*, 1914–15, viii, 97.
5. Quoted from Thomson.
6. von Wasielewski, Th. and Wülker, G., *Beit. z. Arch. f. Schiffs. u. Tropen.-Hyg.*, 1918, xxii–xxiv, 117.
7. Personal communication.
8. Wenyon, C. M., Protozoology, New York, William Wood, 1926, xi, p. 919.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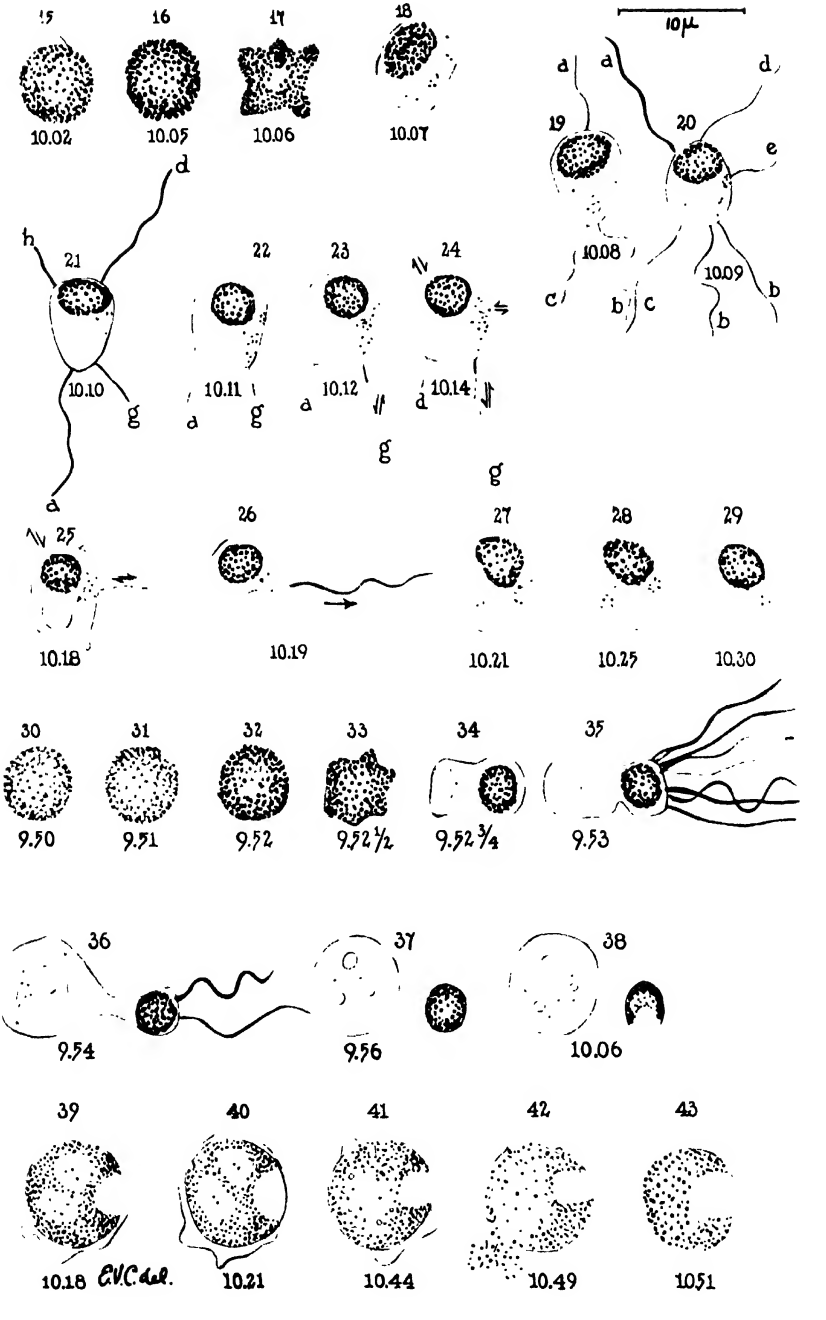
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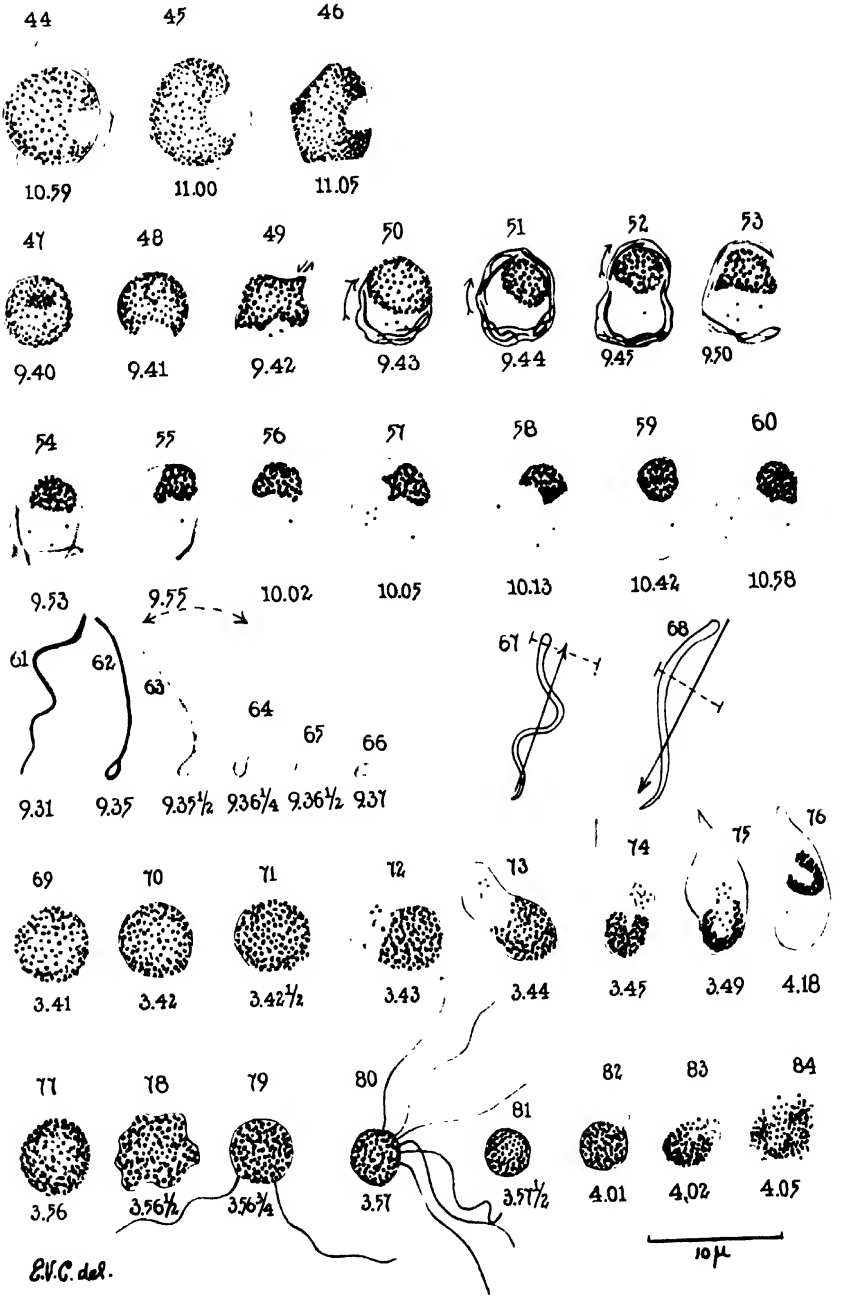
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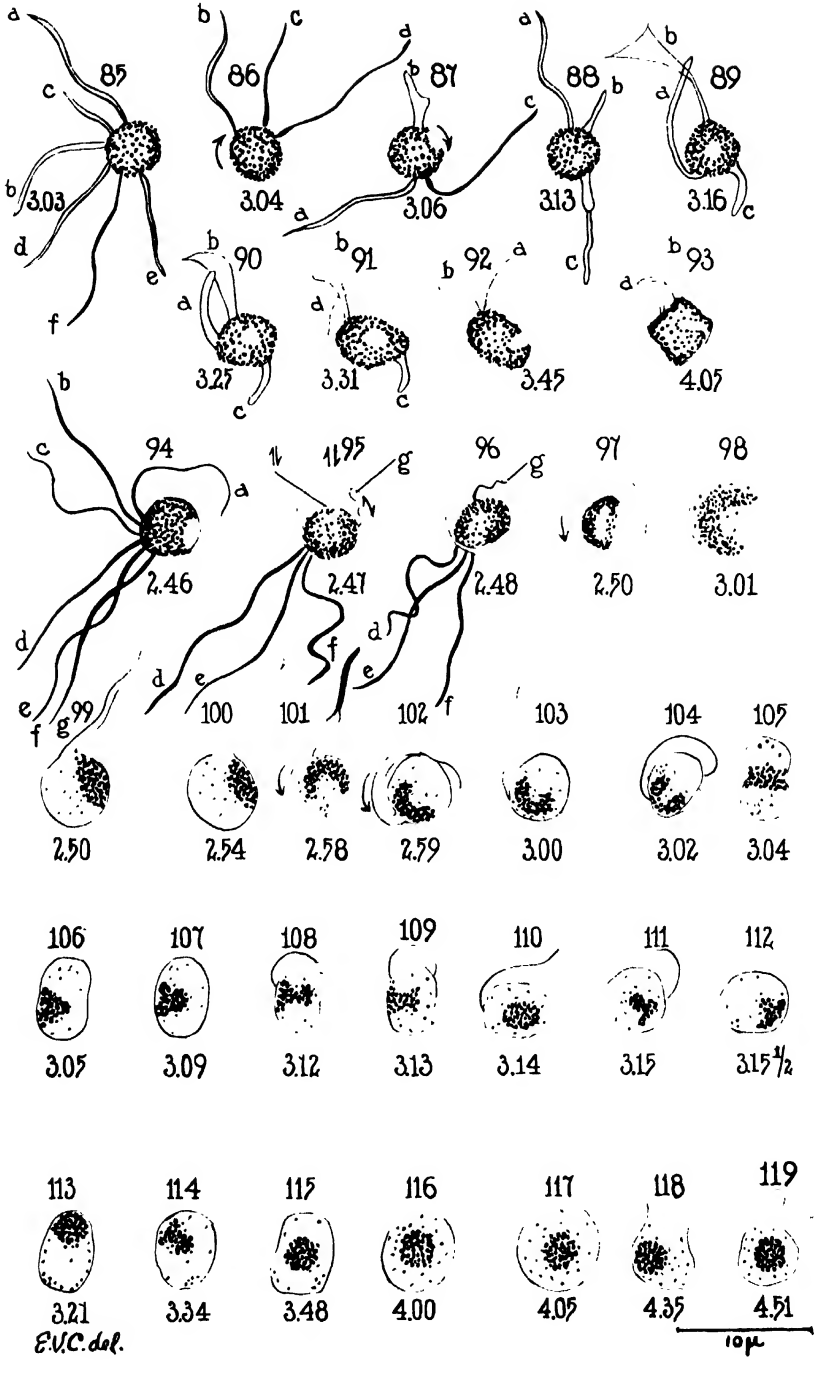
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BONE MARROW.

BY FLORENCE R. SABIN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I. THE VASCULAR SYSTEM. In the analysis of the structure of bone marrow, the most important factor is the nature of its vascular system. Here, in perhaps the last place of the animal organism, the old question of an open versus a closed circulation is still being studied. The matter is, however, clarified over the earlier discussions, inasmuch as we now know that most blood vessels are closed, and now have adequate physiological criteria for the demonstration of an open circulation, as exemplified in the spleen. These criteria are the presence of red blood cells in tissue spaces physiologically, which has a corollary in the invariable occurrence of extravasations in experimental injections, and the presence of a specific muscular mechanism, such as the muscles on the trabeculae of the spleen, for the return of the red cells to the blood stream. These two factors are the essential evidences of an open circulation. A modification of this form has been described by Woollard and Wislocki (1) for hemal glands, in which the red blood cells pass from the vessels into blood spaces, from which, however, there is no pathway and no mechanism for their return into the circulation, so that they degenerate locally instead.

The question of open or closed vessels is important to the understanding of the formation of red blood cells. The entire physiological function of the red cells is within vessels, with the possible exception of the spleen; the concept that they also arise within vessels correlates with the idea of a closed system. For the white cells, the matter is not so significant, since it is generally accepted that they arise outside the vessels, and that they both enter and leave any part of the vascular system by means of their own motility.

The nature of the circulation of the bone marrow has recently been reinvestigated by Doan (2), (3). He has shown that the key to the analysis of the vessels of the marrow is to be found in an experimental reduction to a simplified state. By underfeeding, he was able to

reduce the red marrow of the radius of the pigeon to an extreme hypoplasia, in which the parenchyma consisted of three elements only, blood vessels, fat cells, and a minimal residual framework of reticulum and reticular cells. The general pattern of the vascular system of bone marrow has long been known: it is made by the main nutrient artery with accompanying veins, passing toward either epiphysis and its capillary branches to the venous sinusoids, together with numerous anastomoses of this system with the small vessels of the bone along the shaft and with larger epiphyseal vessels in the mature bone.

Doan demonstrated that the circulation of the avian marrow is closed. He discovered that there is a vastly greater bed of venous sinuses in the marrow than can be patent to the circulation at any one time on account of the rigid confines of the marrow cavity, and that these sinuses, when collapsed, appear as capillaries which are so abundant as to pass between every one or two fat cells. These collapsed vessels can, however, be demonstrated by lines of granules of ink between the two endothelial walls by injections that have been pushed to the maximum without rupture in hypoplastic marrow. Doan analyzed the difference between the injection of these collapsed capillaries and the appearance of extravasation. The importance of the collapsed capillary in physiology was first shown by Krogh (4), (5), who demonstrated that the number of open capillaries in muscle is the register of the amount of function.

In the marrow, the capillaries that lead directly from the arteries of the third and fourth order from the nutrient arteries, making the "transition capillaries" of Doan, are not the fuctioning capillaries, as in other organs, but rather they lead into tufts of sinusoids (figs. 4, 5, 6 in the paper by Doan, 3). These sinusoids make the functioning vascular bed of the marrow; they have walls like capillaries and appear like them when collapsed, but when open they have the diameter and lumen of large veins. The nature of such vessels, having the size of veins but functioning as capillaries, was analyzed by Minot (6) in 1900, who introduced the term sinusoid. The sinusoids of the mesh which happen to be closed at any one time are called by Doan "intersinusoidal capillaries," and it is his concept that there is frequent interchange, a given vessel serving for a time as a collapsed capillary and then as a sinus wide open to the circulation. In other words, in the

functioning bed of marrow, a large part of the vessels must always be collapsed in the space available, while a part are at the same time so widely dilated as to make a zone of sluggish blood flow. The sinusoids which are closed to the circulation at any one time are the actual or the potential site of the formation of the red cells. These collapsed vessels thus become as significant an index of function as the closed vessels of other organs, though in a different and more complex way, since in the marrow the formation of the red cells has to be maintained as well as their circulation. Doan also showed that the number of capillaries in the collapsed phase is greatly increased in gelatinous marrow in association with erythropoiesis and that the collapsed capillaries connect by conical openings with the sinusoids, a point of significance in connection with the method of delivery of the red cells into the circulation. Thus the vascular system of the marrow consists of arteries running longitudinally in the center, and transition capillaries leading to sinusoids, which correspond to the functioning capillaries of other organs. These run transversely. The sinusoids are in part patent to the circulation, in part collapsed and erythrogenic in function. Finally, the sinusoids lead into veins which accompany the arteries.

From the start, the question of an open or a closed circulation for avian marrow was easier to determine than for mammalian forms. In 1881, Bizzozero and Torre (7) described the circulation of the marrow of the bird as a closed type on the basis of the histological structure of the walls of the sinuses and capillaries. They were the first to find that the marrow of the bird could be simplified by underfeeding and that in preparations of such marrow it could be made out histologically that the vessels had intact walls. A closed circulation for avian marrow was confirmed by Denys (8) and van der Stricht (9). Denys (1888) showed that the reason for the difficulty of injecting the vessels of marrow lies in the fact that the circulation is structurally adapted to slow the blood flow; thus he found that a pressure which brings out all the vessels of muscle, fills only the main arteries in marrow. At higher pressure, he found that the sinuses became filled, but the capillaries remained empty, and thus he postulated that there were two types of vessels, one (the sinusoids) that carried blood, and the other (the intersinusoidal capillaries) that carried only plasma.

Concerning the type of circulation in mammalian marrow, there has been less agreement. Of the earlier discussions, the most interesting accounts are those by Bizzozero (10) and Neumann (11), both written in 1869. This was a significant period. The year before, Neumann (12) had announced in one paragraph that red cells arise in marrow, and then both Bizzozero (13) and Neumann (14) found that not only red but also white blood cells are formed there. Shortly before (1862), von Recklinghausen (15) had found that the reaction of silver nitrate demonstrated a cellular lining, endothelium, for lymphatics, and Hoyer (16), in 1865, had applied the method to blood vessels and found their endothelium. Hoyer (17) was, however, unable to silver the sinusoids of the bone marrow, and hence postulated a wide open circulation there. Bizzozero (10) discusses these observations with which he differed, since he could find the endothelial lining for the capillaries and could inject the veins through an interstitial puncture. He saw a distinct border for the sinusoids in many places, and inclined toward the view of a closed circulation, but judged that a satisfactory demonstration of it needed further work. He gave an excellent description of the vascular pattern in the marrow of the rabbit. He described the arteries with their branches coming off at an acute angle, and found that the third and fourth orders lead to the capillaries, and then found two systems of veins, the sinusoids which he terms secondary veins, with their radiating pattern across the marrow, and the true veins accompanying the arteries. He found the capillaries by studying gelatinous marrow. Thus he made many of the observations which have been demonstrated in recent studies, but lacked a concept of the nature of the sinusoid and did not know that the capillaries which he saw in gelatinous marrow could, under other circumstances, be patent sinuses.

Neumann (11) came even closer to the true relation of artery to sinusoid and sinusoid to capillary. He described the arteries as becoming reduced, while still preserving their tunics, to the size of a capillary and then emptying abruptly into large capillaries (the sinusoids). He saw this as a mechanism for slowing the blood flow, and thus had the concept of sinusoidal circulation. He described these capillaries (the sinusoids) as having a diameter six times as wide as an ordinary capillary, and as being lined by a thin membrane.

Finally, he said that at frequent intervals there were little diverticula along the capillary walls from which extended, thread-like, processes or sprouts exactly like the vascular sprouts or *anlagen* which had recently been described for the vessels of the tadpole's tail. As far as we have found, Neumann is the only one who observed these conical openings of the intersinusoidal capillaries, before they were described and interpreted by Doan. In 1877, Langer (18) published a paper in favor of the concept of a closed circulation in mammalian bone marrow. In spite of these observations, Rindfleisch (19), van der Stricht (9), and Muir (20) favored a return to Hoyer's concept of an open circulation.

Van der Stricht (9), in 1892, recognized the significance of the nature of the vessels in marrow to the subject of the origin of red blood cells, and thought that the vascular system and the method of formation of the red cells differed in birds and mammals. In birds he found the circulation to be closed, and that erythrogenesis was intravascular; in the rabbit, on the other hand, he though that the circulation was wide open so that the blood, the mature red cells, wandered freely in the meshes of the connective tissue. Thus he regarded the formation of red cells as extravascular in mammals. A modification of this concept, in connection with the bone marrow of the mammal, is to be found in the work of Maximow (21), who thought that the red cells formed in close contact with the outer wall of the vessels and that from time to time there were local breaks in the continuity of the endothelium which allowed the plasma and the mature red cells to flow out into masses of maturing but still nucleated red cells.

It is now clear that the question of an open or closed circulation in bone marrow concerns the nature of the walls of the sinusoids which represent that part of the capillary bed which is patent to the circulation at any one time; the point at issue is whether they have intact or fenestrated walls. The extreme thinness of the walls of these sinusoids, the variations in diameter of their lumen, and the dense packing of cells along their borders, together with phenomena associated with the opening of hematopoietic capillaries to be discussed later, have constituted the difficulty of this problem.

The whole question of the circulation of mammalian bone marrow has been thoroughly restudied recently by Drinker, Drinker and Lund

(22), and by Doan, Cunningham and Sabin (23). Drinker and his co-workers undertook the study of the method of delivery of the red cells into the circulation in dogs, cats, and rabbits. They developed a carefully controlled method for the perfusion of the marrow with hirudinized blood and with physiological salt solution, and followed the delivery of the red cells into the perfusate. In the animals in which the function of the marrow was at its normal level, there was no increase in the number of nucleated cells put out from the marrow, even with prolonged perfusion; in animals with marrows markedly hyperplastic in the formation of red cells, there was an increase in nucleated red cells. They concluded that immature red cells, which proved to be impossible to dislodge except in the hyperplastic state, were not developing in the pathways of free flow of the circulation. At the close of each experiment, they put India ink into the prefusing solution for a record of the free pathways of the circulation. In these experiments they could show that the circulation in the marrow is through closed vessels. Moreover, they repeated the experiments of Bunting (24) with endothelial toxins, ricin and saponin, and confirmed his observations of the production of hemorrhagic marrow. The same was also shown by Firket (25). The judgment that hemorrhage in bone marrow is a pathological condition is a recognition that a closed circulation is normal, for an open circulation means physiological hemorrhage.

The closed nature of the circulation in the marrow of mammals is also indicated in the work of Doan *et al.* (23) in injections of the marrow of rabbits from which the granulocytes had been removed by the injection of large doses of inactivated typhoid bacilli; in these preparations the evidence of intact vessels was convincing.

The question of an open circulation has, however, been revived by Jordan and Baker (26) for amphibian material. They have shown that erythropoiesis in the frog takes place largely in the spleen, except for a short period after hibernation, when the bone marrow becomes somewhat involved; but after splenectomy, there is a marked transference of erythropoiesis to the marrow, making favorable material for study. They made injections with a carefully controlled technique, introducing only a small amount of ink into the heart, while withdrawing a compensatory amount of blood from a vein and allowing the

pressure from the heart beat to carry the injection mass into the marrow. It is now clear that with an open circulation the fenestrae in the walls of the vessels can be seen with the microscope, as was demonstrated by Mollier (27) for the ampullae of the splenic arteries and the walls of the veins (sinusoids) of the splenic pulp, and that from such vessels both plasma and cells flow out physiologically, as does water through a sieve; in other words, an animal bleeds into its own spleen and an open circulation is there plainly and unequivocally demonstrable at blood pressure. Jordan and Baker describe a variation of such an open structure for the walls of the sinusoids in amphibian marrow, namely, that the endothelium fades off into a reticulum, leaving no definite walls for the vascular channels, a condition which would call for a wide open circulation physiologically. Their figures of injections are, however, not convincing in that they are not comparable to those that are obtained by a single spurt of ink into the splenic artery at blood pressure and not comparable to hemorrhagic mammalian marrow. There is, of course, one striking contrast between conditions of spleen and bone marrow; the latter is not an extensile organ, so that it might be postulated that the fat cells could be packed in marrow so densely as to offer resistance to the spread of an extravasation; but in an organ that is non-extensile, it is difficult to imagine what mechanism could bring about the return of the inert red cells to the circulation. To sum up the question of the nature of the circulation in bone marrow, the balance of evidence at the present time is toward a closed type. The significant points in the structure of the vascular system are that the circulation is sinusoidal, that is, adapated to a sluggish flow, and that a large part of the sinusoids at any one time are collapsed and erythropoietic in function.

II. Distribution of red, fatty and gelatinous marrow. The question of the distribution of red marrow is an important one and varies markedly in different animals. As is well known, in the larger mammals and in man, the marrow of the long bones of the adult is predominantly yellow, and thus there is a large reserve potentially available for the formation of blood cells, but not used under normal conditions. The best account of the distribution of red marrow in the human being is that of Piney (28). He found the bone marrow at birth to be of a pink rather than a red color; by the age of seven, there

is some fat in the marrow; between twelve and fourteen years, there appears a definite macroscopic patch of fat in the middle of the shaft of the femur, which gradually extends until in the adult there is only one patch of red marrow left near the upper end of the diaphysis. The same process takes place in tibia and fibula, in humerus, radius, and ulna, only more rapidly and completely. The epiphyses also become entirely fatty, as do the bones of carpus and tarsus. Red marrow is to be found in the ribs, vertebrae, sternum, in the bones of the skull and in the os innominatum.

In human pathology this distribution is made use of by selecting for routine study a bone in which the formation of blood takes place normally, usually a vertebra, less often a rib, to determine any lowering of activity; and a long bone, usually the shaft of the femur, to detect any extension of hematopoietic territory. For human biopsies the center of the tibia (Ghedini, 29, 30; Morris and Falconer, 31; Caronia, 32; Kramár and Hensch, 33; Peabody, 34) or the sternum (Seyfarth, 35; Yamamoto, 36) is used. Kramár and Hensch have applied the technique to the infant. In the dog and the cat the long bones are occupied by red marrow but with spicules of bone. With rabbits and guinea pigs the cavity of the long bones is involved in blood formation, and since there are no spicules of bone, these animals afford excellent material for study, as the difficulties of decalcification may be avoided. In birds the humerus is filled with air for lightness, and, as shown by Meltzer (36A), for respiratory function as well; but the radius and ulna are normally filled with red marrow; spicules of bone are sometimes present, but at times are entirely absent; in starvation, when the marrow of the radius has become entirely hypoplastic, there may be some compensatory increase in red marrow in the femora.

The significance of the fat in marrow, both the amount that is present in red marrow and in marrow that is normally wholly yellow, has long been known, but the rapidity with which the fat can be mobilized out of the marrow is only to be realized through experimental methods. In his experiments with induced hypoplasia in the pigeon, Doan (3) underfed a bird until it had lost 150 grams; he then removed a small bit of marrow, under ether, from the radius at biopsy, and found that it had been reduced to the extreme fatty form; then the animal was fed, and in twenty-four hours, as shown by a second biopsy

from the same bone, there were marked changes in the fat cells, while in forty-eight hours, when the pigeon was killed, the fat had almost entirely disappeared and the marrow had become gelatinous. The process is as follows: the fat cells first return part way toward their embryonic state. In the development of a fat cell, the lipoid is first laid down around a centrally placed nucleus in small droplets. These gradually fuse into larger and larger droplets, until finally the entire content of fat is in one large sphere. In the regression of the fat cell, the first change is the splitting up of the large sphere of fat into small droplets; the nucleus rounds up as if released from pressure; the droplets of fat then start to pass out of the cell on the side toward a patent sinus, leaving branched processes of cytoplasm to mark this shrinkage. The meaning of these changes in the fat is that the cavity of the bones is entirely filled with tissue, excepting the pneumatic wing bones of birds, and when there is any increase of cells in marrow, something has to pass out to make room. For acute expansion there are only three things which can pass out, namely, the fat, the blood circulating in the vessels, and the newly developing marrow cells. The fat cells make the main regulatory mechanism for space available to new marrow cells when there is any marked change in the amount of formation of blood, and on this account the fat in marrow is in an extremely labile state. The fact that the entire fat can be demobilized from the bone of a bird in forty-eight hours is evidence of this. In the study of tubercular rabbits, Doan and I have seen large masses of fat droplets in fresh specimens of peripheral blood at a stage when a marked anemia and leucopenia were correlated with a massive development and encroachment by tubercles in the bone marrow. It has been shown by Bloor (37) that there is an increase in unsaturated fatty acids in the blood after hemorrhage. Thus the histological and chemical evidence is toward the view of a shifting of the fat from the marrow cavity, when increased formation of cells is demanded.

When the fat disappears so rapidly from the marrow, its place is not at once completely occupied by blood-forming tissue, which has no such speed of regeneration, but Doan has shown that its place is rather immediately occupied by a gelatinous substance which fills in all the spaces left by the shrunken fat cells. Thus, fasting gives a fatty marrow; feeding after this induced hypoplasia gives the following sequence of events: removal of fat, gelatinous marrow, erythropoiesis.

Gelatinous marrow has long been known. It was described by Bichat (38) in 1801, who saw it in certain diseases, for example in rickets, and it is well known to pathologists. Neumann (11) and Bizzozero (10) both described gelatinous marrow in 1869, and the latter noted that it often occurred in young animals and was often red in color. In 1881 it was shown by Bizzozero and Torre (7) that it could be produced in birds by underfeeding; while in 1904, Jackson (39) repeated these experiments for the pigeon and demonstrated the same for the rabbit. Its frequent occurrence under pathological conditions has led to the universal view that it is a form of degeneration. Neumann (40) described it as a transformation into a myxomatous tissue in which the fat cells become changed into branching mucoid cells, lying in a homogeneous matrix. Muir (41) noted again that gelatinous marrow might have a red color, which was confirmed by Dickson (42). Muir found that the gelatinous substance is an albuminoid and believed that it came in part from the fat. Dickson regarded the gelatinous stage as a form of degeneration, but he noted that in gelatinous areas the vascular channels often remained patent to circulation to a much greater extent than might be expected in areas of degeneration. These observations of Bizzozero, Muir, and Dickson can now be interpreted in the light of Doan's observations that erythropoiesis begins in the gelatinous marrow after hypoplasia. Stefko (43) recently had an opportunity to study the marrow from sixty-three human cases of starvation and found gelatinous marrow as characteristic. The source and the nature of the gelatinous substance are not, as yet, known.

Beside the mechanism for shifting fat out of marrow, there is another means of increasing space which can be used under extreme need. Drinker *et al.* (22) found that there is a decrease in the spicules of bone in the marrow of the tibia of dogs after repeated hemorrhage; and Johnstone (44) has described an enlargement of the marrow cavity through an actual thinning of the compact bone of the border of the ribs in the dog during a phase of increased formation of red cells after splenectomy.

It is clear that both the wholesale removal of the fat and even more the removal of bone represent mechanisms in conditions that call for a change in the level of blood formation from the normal. The physiological shifting out from the marrow of each successive generation of

mature red cells and white cells in the normal function of the organ is, on the other hand, more probably compensated for by vasomotor regulation.

Yellow marrow is at all times a potential reserve for blood formation; there is some evidence that it may also be a special factor of safety in infections. Infection of red marrow more often than of yellow was indicated in typhoid fever by Quincke and Stühlen (45) and Fränkel (46), (47); Müller (48), in a series of cases of influenza, found that the red marrow had been invaded by organisms and damaged enough to cut down granulopoiesis, while the yellow marrow had escaped the infection and was becoming active. Hartwich (49), (50) has not found that yellow marrow infected less often than the red; but Müller (51) reported the vertebral marrow involved in over 80 per cent of a series of different infections and the marrow of the long bones in 25 per cent. Thus, though the evidence is conflicting, the structure of yellow marrow with its high proportion of collapsed capillaries might be adapted to give a smaller dose of organisms per unit area.

III. FORMATION OF RED BLOOD CELLS IN MARROW. The structure of marrow with relation to the formation of blood cells was not analyzed in the active state, for so crowded are the cells that their relationships are diffcult to determine. To become familiar with the process, it was necessary to begin the study of the formation of blood cells in the early stages of recovery from the hypoplastic state, and thus to determine methods to make marrow inactive and then to stimulate it at will.

Erythropoiesis in avian marrow. The crowding of cells is far less in avian marrow than in mammalian, and hence the general agreement that erythropoiesis takes place within the vessels in the bird (Bizzozero, 52; van der Stricht, 9; Muir, 40; Danchakoff, 53). The meaning of this intravascular origin of the red cells in the bird was made clear by the observations of Sabin (54), in studies on the living chick blastoderm of the second and third days of incubation: this study proved that red cells arise within vessels because they come from endothelium, or that the power to synthesize hemoglobin develops in the angioblast and its derivative, endothelium. It was then shown by Doan (2), (3), (23) that endothelium is as fundamental to the formation of red cells in the adult bird as during the stages of incubation. As has been described, after the period of underfeeding the marrow was fatty, and

forty-eight hours after feeding was begun the fat had been removed and the marrow had become gelatinous.

The next phase in regeneration, after the gelatinous state, was erythropoiesis alone, so that there was a chance for the study of this process completely separated from granulopoiesis. In the gelatinous marrow the dense capillary plexus was obvious both on account of the closing of many of the patent sinuses, as contrasted with the normal state, and because of the metaplasia and swelling of the endothelium. There is an important physiological condition to be considered when so large a proportion of the sinusoids is collapsed, because the entire marrow has then a low blood supply, or is then in a state of low oxygen tension. This point is of great significance for erythropoiesis. It is in agreement with the observations of the increased formation of red cells in high altitudes (Bert, 55; Viault, 56; Douglas *et al.*, 57; Barcroft *et al.*, 58;) and with the work of Brill (59) on low oxygen as a stimulus to marrow, and of Dallwig, Kolls and Loevenhart (60) on increased erythropoiesis in animals kept in an atmosphere of low oxygen tension. Jordan and Speidel (61) have shown the same thing in amphibian material, expressing it in terms of the stimulus of CO_2. Campbell (62), (63) has shown not only that low oxygen tension increases the formation of red cells, but that high oxygen decreases their formation. Moreover, during their increase there is a change in the color index.

It should be noted that the point that low oxygen tension favors the formation of red cells is not contradicted by the well known fact of erythrogenesis in the spleen, inasmuch as the blood of the spleen is predominantly venous and relatively stagnant, as compared with the circulation in other organs. It is clear that the conditions in the spleen are adapted to the formation of both red and white cells; the sluggish blood flow gives low oxygen tension for the red cells and brings the maturation factors in right concentration for the white cells.

The first step in the formation of red cells is the swelling of some of the endothelial cells of the capillaries; then follows the division of the swollen endothelial cells. We have now been able to see that this swelling or phase of growth actually precedes division, by watching the process in the vessels of living chick blastoderms growing in a solution of liver extract, for which we are indebted to Dr. George Minot and Dr. Edwin Cohn.

In avian marrow, division of these enlarged endothelial cells was found with the equator of the spindle parallel to the wall of the vessel; when the equator of the spindle is perpendicular to the wall of the vessel, two endothelial cells result; when parallel to the wall, the outer cells become the endothelial wall and the inner ones megaloblasts.

In the next stage after the swelling of the endothelial cells of the intersinusoidal capillaries, the capillaries become even more prominent in the depleted marrows by the development of lines of megaloblasts within them. The megaloblast is the first stage in the maturation of the red blood cell. It is readily discriminated in sections by the large vesicular nucleus, the characteristic large nucleolus, and the scanty chromatin; the amount of hemoglobin in the megaloblast is so slight that it cannot be seen at all when the cell has been stained in any basic dye, since the basophilia of the cytoplasm is so strong that it obscures the weak acidophilia contributed by the hemoglobin. In fresh preparations, on the other hand, a minimal amount of hemoglobin can be detected and the cell has large numbers of mitochondria. It is discriminated also by an extreme grade of the phenomenon of reticulation. (These supravital reactions are shown in figures 44 and 58, plate IV, in article by Doan *et al.* (23).) The origin of megaloblasts from endothelium of the intersinusoidal capillaries in bone marrow of pigeons at different periods of regeneration has been confirmed by Muller (64).

As erythropoiesis proceeds, the megaloblasts give rise to early erythroblasts; then late erythroblasts develop, and finally the nucleated red cells of the adult avian type (Sugiyama, 65). In the bone marrow the megaloblasts remain against the endothelial wall and the older types are thus to be found in the center of the capillaries which dilate to meet the needs of the increasing number of cells (fig. 6, in Doan *et al.*, 23). This arrangement was illustrated by Denys (8) in 1877 and then by van der Stricht (fig. 50 on plate X of van der Stricht's paper, 9), who introduced the term of hematopoietic capillaries for such vessels.

Erythropoiesis in the mammal. In spite of the complexity of mammalian marrow, due to the crowing of the cells, the same fundamental structure can be determined. In the mammal also the red cells arise within vessels because they come from endothelium (Doan *et al.*, 23), and the white cells arise in the intervascular spaces. The methods for

depleting and stimulating mammalian marrow are more complex and the study of the control by diet has only begun (Whipple *et al.*, 66; Robscheit-Robbins *et al.*, 67; Murphy and Minot, 68; Minot and Murphy, 69).

In 1901 and 1902, Muir (41), (70), who had found that the red cells of avian marrow develop within vessels, observed that a separation of the formation of red and white cells could also be seen in mammalian marrow in areas in which red marrow was extending. He described the white cells beginning near patent sinuses and nucleated red cells first appearing in lines or small clumps. The concept of erythrogenic and leucogenic centers in mammalian marrow was still more clearly formulated by Selling (71), (72), who saw that after poisoning with benzol the regeneration involved two types of islands, erythroblastic and granuloblastic, and that each remained separate and was made up of one strain for some time. Then Bunting (24) found that though it might be difficult to demonstrate the two types of centers as distinct in active marrow, nevertheless, they could be unmasked by drawing out the excess of myeloid cells with aleuronat. He, however, accepted the general concept that both types of centers were extravascular in the mammal.

Cunningham and Doan (73) showed that erythropoiesis could be completely unmasked in the rabbit by repeated intravenous injections of inactivated typhoid bacilli; these experiments were extended (Doan *et al.*, 23) by more elaborate methods for studying erythropoiesis. Since it was known that bleeding gives a hyperplastic marrow, while transfusions lower the rate of erythropoiesis, a series of matched donors and recipients were used for repeated transfusions, and in these two groups Doan and his co-workers studied the formation of the red cells in active and quiescent state after withdrawing the myeloid elements as before, namely, by injections of inactivated typhoid bacilli. In these experiments the swollen endothelial cells of the intersinusoidal capillaries were clearly demonstrable as the first step toward erythropoiesis, after a state of hypoplasia. This swelling of the endothelial cells has been seen before and was described by Maximow (21) in 1909, without, however, the same interpretation. The relation of the megaloblast, as the first step of erythrogenesis, was also shown to be as true for the mammal as for the bird. As erythropoiesis proceeds

in the recovery from hypoplasia, erythroblasts develop from the megaloblasts and form the center of small groups, the megaloblasts always retaining their original position against the endothelial wall (fig. 36 in article by Doan *et al.*, 23).

In normal mammalian marrow megaloblasts are either absent or present in but small numbers, so that it is the early and late erythroblasts that form the edges of the groups of developing red cells with normoblasts in the center of the lumen of the capillaries, which dilate to accommodate the cells as they increase in numbers. Along the edges of such clumps, endothelial nuclei, sometimes swollen, can be made out, but as the process continues the endothelial cells are stretched until they become very thin. This concept of the normoblasts being in the center of the clumps is the reverse of the description given by Bunting (24), who interpreted the erythrogenic clumps as having a border of normoblasts around clumps of immature red cells. In the early stages of regeneration, as well as in the unmasking of normal erythropoiesis, both erythroblasts and normoblasts are often found in single rows, filling the lumen of a capillary, and these single rows of developing red cells, outlining the regular pattern of a vascular network, are convincing evidence of their intravascular origin and development. In sections of active bone marrow, the myeloid cells often seem to be resting directly on the clumps of intravascular red cells, which is inevitable with the great crowding of the cells, and hence, to see clearly the intravascular position of the immature red cells, one must resort to methods for unmasking erythropoiesis by depleting marrow of its myeloid elements.

The concept that the immature red cells develop in erythrogenic capillaries gives a clear idea of how the inert red cells get into circulation. It is obvious that the red cell does not enter by its own motility. Under the prevailing theory that the inert red cells originate outside vessels, it has been thought that either the circulation of mammalian marrow is always open, or that as the red cells develop against the outer wall of the sinusoids, they bend and break the thin walls (Maximow, 21). It was then postulated that a growth force pushes the red cells through constant or temporary openings into the blood stream. If, however, the concept that the developing red cells are within capillaries, temporarily closed to the circulation, proves to be correct,

then it is easy to see how these capillaries, when distended with normoblasts, might open up enough first to let the plasma in between the normoblasts and then to sweep these capillaries empty of their newly formed red cells. Such an opening of closed capillaries would be entirely analogous to the proved opening of collapsed capillaries elsewhere in the vascular system (Krogh, 4; Hooker, 74, 75; Rich, 76; Richards, 77; Richards and Schmidt, 78; Bayne-Jones, 79). It has been shown by Key (80) that the immature red cells have a quality of stickiness in their surface membranes which disappears just as the normoblast becomes the red blood corpuscle. The exact stage of the loosening of the clumps of normoblasts by the plasma filtering in around them has been demonstrated by Doan *et al.* (23; see their fig. 35), who unmasked erythropoiesis in the rabbit and then injected the marrow with India ink. The sinusoids and their erythropoietic capillaries could then be seen with continuous and intact endothelial linings, because there were no myeloid cells along the outer border; in these specimens the ink from the sinusoids could be seen as it had just entered the hematopoietic capillaries, and was found sticking to the normoblasts in their lumen. A similar experiment, but without the unmasking of the vessels, was made by Drinker *et al.* (22). They took a rabbit in which, after repeated bleedings, the marrow was in an active state and selected a time when the peripheral blood showed a cycle of delivery of both red and white cells into the circulation, and immediately made an injection with India ink. In this stage there were places of irregularly outlined carbon, apparently extravasation, which, however, were not general, but rather sharply localized, so that they described the ink as being held between columns of cells upon which it seemed to border. Lacking the depletion of the myeloid elements, the endothelial boundary enclosing the young red cells was not so obvious, but their figure (fig. 37 in Drinker *et al.*, 22) can be interpreted as the entrance of ink into the intersinusoidal capillaries just opening up to the circulation, and is in striking contrast to their injections of hemorrhagic marrow after saponin (figs. 43 and 44).

The concept of the opening of erythrogenic capillaries at certain periods to admit the maturing red cells to the circulation gives a feasible explanation of normoblastic crises from a premature opening of such capillaries to the circulation. Under conditions of extreme

need for the regeneration of red blood cells, megaloblasts can also be found lining the walls of patent sinuses.

After their studies of unmasked erythrogenesis in the rabbit, Doan *et al.* (23) also found that the same processes of regeneration from hypoplasia could be traced in occasional opportunities in human marrow, reporting a case of typhoid fever, one of starvation in the insane, and two of bacterial endocarditis.

The process of the origin of red cells from endothelium was then studied in the human by Peabody (81) in a series of cases of typhus fever and illustrated from one case in which the preservation of the tissue and the technique of preparing the serial sections were faultless. Peabody brought out that the pathologist, as a matter of fact, has relatively frequent opportunities to study erythropoiesis alone in human material, under conditions when the marrow of the long bones, normally aplastic, is beginning to take on hematopoietic function. This occurs not only in recovery from anemia, but also with most infections and intoxications (Naegeli, 82). Muller (83) was able to produce an hypoplastic anemia in rabbits by collargol and by regulating the doses and varying the time of killing the animals after the last dose, she obtained material both in the aplastic state and in the phases of regeneration of both red and white cells. She also showed (84) that with injections of India ink there was such a stimulus to the formation of red cells that the open sinuses were lined with nucleated red cells. To this material, Doan and Sabin (85) have added the study of the recovery from anemia of a series of tubercular rabbits. They found that after massive doses of bovine bacilli, the marrow became simplified, that is, relatively aplastic for blood formation, on account of an extensive encroachment with tubercles, but that the marrow tended to recover in every case in which the animal survived the acute phase of the disease, regardless of the extension of the tubercular processes elsewhere. The platelets started to regenerate first, then the red cells, followed by the granulocytes.

In recent experiments with amphibian marrow, Jordan and Baker (26) have found the same opportunity to study erythrogenesis alone, since the stimulus in the frog's bone marrow after splenectomy is limited to the production of red cells. It is also interesting to note that Koolman (86) reports that the removal of the bone marrow is a stimu-

lus to regeneration and that in the stage of recovery after removing the radial bone marrow in dogs, the red cells began to rise in the circulating blood, while the white cells were still falling.

In all this material, the avian, the experimental mammalian, and the human, it is evident that the initiation toward regeneration from aplasia begins with erythropoiesis, rather than with an equal start in the production of red and white cells. Thus, beginning with the earliest stages of regeneration from a hypoplastic state, it is possible to study first erythropoiesis and later granulopoiesis in separate foci. Where erythrogenesis is thus simplified, it can be seen to be intravascular in type, with a swelling of endothelium as the preliminary phase. There is one dissenting opinion on the interpretation of such experimental material. Jordan and Baker studying such simplified erythropoiesis in amphibian forms, interpret the thin cellular borders which they also found around developing red cells as made by flattened reticular cells having no relation to endothelium. With this exception the more recent workers with simplified material (Doan, Cunningham, Sabin, Peabody, Muller) agree that the evidence is for the origin of the red cells from endothelium and the intravascular maturation of red cells in mammalian material.

It thus appears that the observation of the presence of erythrogenic and leucogenic centers in marrow, starting with Muir, Selling, and Bunting, finds its interpretation in the concept that the red cells develop in erythrogenic capillaries closed to the circulation; that the complete separation of the two processes, one within vessels closed to the circulation, and hence in places of low oxygen tension, and the other between the dilated sinuses and thus provided with an abundant and sluggish flow, can be demonstrated both in the recovery from aplasia and by the unmasking of normal erythropoiesis by methods which remove the excess of myeloid elements. When one has become familiar with such material, the same structural relationships can be seen in normal and even in hyperplastic marrow, in spite of the crowding of myeloid elements around the erythrogenic capillaries. It is important to note that the red cell is the first of the blood cells to form in the embryo and throughout life the regeneration of red cells precedes regeneration of the white cells from an aplastic state of bone marrow.

IV. FORMATION OF WHITE BLOOD CELLS IN MARROW. *Maturation.* It is known that granulocytes, that is, neutrophilic, basophilic, and eosinophilic leucocytes, arise outside blood vessels and pass into them by their own motility. The cell from which they come is a matter on which there is difference of opinion. According to one concept of the origin of blood cells, there is a common stem cell for both red and white strains, which is a basophilic, polyvalent form. This hypothetical cell has had many names (the mesamoeboid cell, the lymphocyte, the lymphoidocyte, the hematoblast, the hematocytoblast, etc.), resulting in a condition in which the nomenclature has become more complicated than are the facts.

While a science is in a descriptive phase, complex terminologies arise and can be carried, but as a subject becomes experimental, it is essential to have criteria that can be readily compared. To this end the factors of maturation of granulocytes have been expressed in a diagram. On chart 1 maturation is represented as a series of varying factors, constantly changing; in some levels, the factors may increase together, in others some may increase while others decrease. The cells are shown in three levels, the original non-granular stages, the granular marrow cells or myelocytes, and the leucocytes, the three levels being separated by two critical points, shown as solid vertical lines. The first marks the point of the beginning of granulations and the second that at which the last cell division of the myelocyte has taken place, with the reduction to a cell of common size and state of maturity, the definitive leucocyte.

Level I. The cells of level I can best be studied in simplified marrow. In completely aplastic adult bone marrow, such as that of the pigeon after a short fast (3), from which active marrow is readily and quickly regenerated, only three types of cells can be seen: fat, endothelium, and reticulum. Of these three, the fat is but accessory to blood formation. Evidence has just been given that the red cells regenerate from the endothelium. The white cells come from the reticulum.

The reticular cells are easily found both in fresh and in fixed aplastic marrow, since they are the only type in the interstices between the fat cells. They are few in number and are scattered along the reticular framework. Their lack of differentiation gives few characteristics by which to describe them. The cytoplasm is faintly basophilic and

shows no granulation, not even mitochondria; if mitochondrial substance is present, it is too finely divided to be seen; the nuclei have so little chromatin as to make them scarcely more basophilic than the cytoplasm. These cells, the so-called reticular type, are probably as close to primitive embryonic mesenchyme as occurs in the adult animal. In this sense, the term reticular cell is used for a type less differentiated than the fibroblast (Mallory, 87), but as a form which

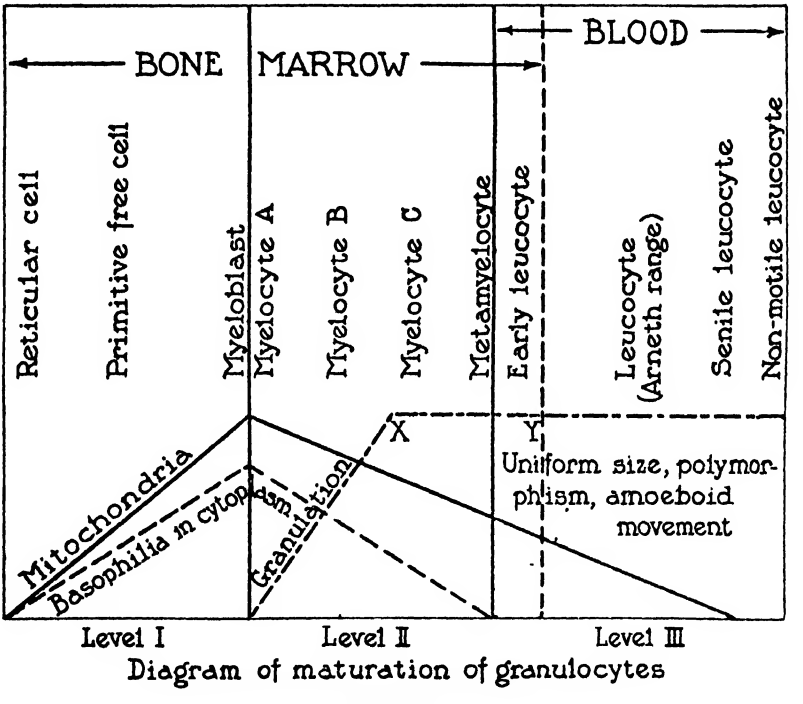

CHART 1.

may also differentiate into a fibroblast. Their characteristics and their relation to the origin of the white blood cells were described by Cunningham *et al.* (88) and illustrated as individual cells in their figures 2 to 5, plate I. They are well shown by Orsós (89) in his figures 1 and 2, in their relation to the reticular fibrils in the interstices between fat cells in sections of human marrow from a case of aleucemic leucemia. These cells are not an hypothetical form; they can always be found in bone marrow, lymph glands, and spleen. They have been described

by Lang (90) as cells of embryonic type along blood vessels, in contrast to the adventitial cells of Marchand, the so-called phagocytic clasmatocytes. Both the reticular cells and clasmatocytes can be found along the blood vessels in the connective tissues, and both have been illustrated by Kubie (91) along the vessels of the brain. He found the reticular cell as a normal type in especially large numbers along the vessels of the nervous system and used the term lymphoblast, because in this location they are the forerunners of the lymphocytes in the perivascular reactions of the nervous system. The above observations demonstrate and show how to find the actual undifferentiated reticular forms which are the type long postulated by hematologists as the forerunners of the blood cells.

In regenerating marrow, this primitive reticular cell, with repeated divisions, gives rise to a primitive free cell. In this process a few mitochondria are elaborated in the cytoplasm and a basophilia is gradually built up. The stage of the primitive free cell is shown by Cunningham *et al.* (88) in their figures 6 to 10, and by Orsós (89) in his figures 3 and 4. Finally, as the basophilia of the cytoplasm and the numbers of the mitochondria reach a maximum, the cell becomes the myeloblast (chart 1).

Naegeli (92), to whom is due the concept of the myeloblast, does not so limit the term to the stage of full maturity in this level; but the convenience of such a limitation of the term lies in the fact that this is the cell of myeloblastic leucemia. Since this period is one of growth and division, as well as one of maturation in regard to mitochondria and basophilia, the cells are to be found in varying sizes; but in normal bone marrow, it is the primitive free cell of small size, similar to the small lymphocyte, but differing from it in having fewer mitochondria and a less differentiated nucleus, which is to be found in the largest numbers. It is this small cell which has been confused with the mature small lymphocyte, which has been so great a stumbling block in the understanding of the mechanism of bone marrow. The reason for the difficulty is clear, for the morphological points of distinction between the primitive free cell and the small lymphocyte are not striking; it is now certain that lymphocytes may arise in bone marrow both under normal and under pathological conditions, but the usual cellular output of the marrow is limited to red cells and the three

strains of the granulocytic leucocytes, so that the primitive free cell of bone marrow normally produces granulocytes in that location.

Level II. The classification of the myelocytes, level II, by the number of the granules was introduced by Sabin, Austrian, Cunningham and Doan (93) from a study of leucemic blood. Myelocyte type A is derived from the myeloblast, of maximum basophilia and mitochondria, by the addition of the first small clump of granules; type B represents the stages during which granulations are increasing in number; while type C is the stage when they have reached their maximum. These three stages are readily discriminated in any technique, but the earliest granulations, as soon as they are large enough to be visible, are more readily seen in fresh, unstained marrow or after supravital staining than in fixed films, where they may be obscured by the marked basophilia of the cytoplasm. However, with careful technique, they are visible in methylene-blue-azur, as shown in Pappenheim's *Atlas of Myeloid Cells* (94). The conventional classification of level II is promyelocyte and myelocyte, based on the changing chemistry of the granules; the relative value of the two classifications will be discussed later in relation to their use in the study of marrow as an organ.

During the stages of level II, the basophilia and mitochondria built up in level I gradually disappear as the granules increase in number. The vertical line at the end of level II is based on one point, namely, on the last cell division. Around the second critical line in chart I, at the end of level II, two factors vary, the cessation of the basophilia and the beginning of ameboid activity; both are shown at the line merely for the sake of simplicity. In general, the basophilia does disappear at the end of level II, but a slight basophilia may be carried over to the early leucocytes normally and, in conditions of leucocytosis, where young leucocytes are drawn quickly into the blood stream, as in pneumonia, the young leucocytes of the the blood are often still basophilic.

Concerning the onset of the ameboid movements, the term metamyelocyte is convenient; the myelocyte C shows no movement of the granules in the fresh state; the cytoplasm is probably too much of a gel to allow of the streaming of the granulations, which makes possible typical ameboid locomotion. Myelocyte C may show slight locomo-

tion, but with no such speed as that which characterizes ameboid movements. The stage of the metamyelocyte from human blood was shown by the writer (Sabin, 95), under the mistaken label of "large mononuclear" in supravital reaction. It is frequently found in small percentage in the circulating blood of people practically normal, as confirmed by Shaw (96) and Stetson (97). Nevertheless, it is a cell of the marrow and its presence in the blood probably indicates some absorption of bacterial products. The very beginning of ameboid activity is often seen in the metamyelocyte as a slight local shifting of the granules, as the cytoplasm starts to become more fluid; this property, together with a slight indenting of the nucleus, discriminates the type from myelocyte C, but the larger size separates the cell from the leucocyte and possibly it is the stage just before the final division. In the leucopenia of typhoid fever, the myelocyte B may skip the stage of myelocyte C, and still having marked basophilia, but deficient granules, may begin to show streaming of granules and appear in the circulating blood. Thus there are normally slight variations in the point of the disappearance of the basophilia and the onset of ameboid movement around the line at the end of level II, which may become quite wide variations under abnormal conditions.

As is shown in chart 1, the mitochondria gradually diminish in number; at the same time they also become smaller, and finally disappear entirely at the stage of senility. Throughout level II there are both growth and cell division, so that there is variation in the size of the cells with a tendency for myelocytes C and metamyelocytes to be large forms.

Level III. The third level, marking the end of maturation, is characterized by cells of approximately uniform size and content of the specific granulation. These factors mean functional maturity. The uniformity of size is due to the cessation of division which precedes the stage of polymorphism; the nuclei fragment and the cell frequently drops off bits of cytoplasm, but division no longer takes place. As is well known, ameboid movements are a constant phenomenon of the leucocytes, whenever they have a surface to move upon. The leucocytes become more mature in the circulating blood according to the Arneth pattern (98), involving the nuclear changes, and finally pass into the non-motile phase (Sabin), in which the granules no longer

stain supravitally in neutral red; the cells in this stage are the fragile forms with easily ruptured membranes in fixed films. Cells in the non-motile phase appear in the blood in showers (Sabin, Cunningham, Doan and Kindwall, 99), indicating that leucocytes in part die in the blood stream. In part they pass out into the tissues, a point which will be discussed later.

Chart 1 is based on cytoplasmic criteria. There are also nuclear criteria. In general, immature phases of both the red and the white strains of cells have less chromatin and possess true nucleoli, plasmasomes, consisting of pyronin (Schridde, 100) rather than condensations of chromatin. With this exception, differences in nuclei are more sharply differential for discriminating the erythroid from the myeloid strains (Türk, 101), and for separating myeloblasts from lymphoblasts (Schridde, 100), than for tracing the stages of development within a single strain.

Relation of granulocytes to the vascular pattern. The process of the regeneration of the white cells in aplastic marrow is different in place, in time, and in method from that of erythogenesis (Doan *et al.*, 23). In the earliest stage of regeneration of red cells, the entire gelatinous marrow is seen to have a low blood supply with but few patent sinuses and many collapsed ones. Along the border of such a marrow there is a narrow zone, where the blood vessels of the marrow anastomose with those of the shaft of the bone. As granulopoiesis begins, every vessel of this narrow border is to be found patent to the circulation, that is to say, there are no collapsed intersinusoidal capillaries. No other area of the marrow has so constantly a maximum blood supply, and it is in this narrow strip that the onset of regeneration of white blood cells is to be found. The contrast between the central, relatively avascular, erythropoietic zones and the marginal, vascular, granulopoietic zones is well shown by Doan *et al.* (23) on their plate II. In this area can be followed the stages of maturation, through the three phases or levels just described—the primitive, the myelocytic, and the leucocytic.

In their relation to blood vessels, it will be seen that in granulopoiesis the conditions are the exact reverse of erythrogenesis. With the red cells the most immature forms are against the endothelium on the inside of the vessel; with the leucocytes, on the other hand, it is the most mature forms, myelocytes C, the metamyelocytes, and the

leucocytes, that border vessels, and the metamyelocyte and still more the leucocyte that enter the lumen.

In the beginning of regeneration from the aplastic state, the difference in time for the regeneration of red and white cells, and the difference in place allow for a separation of the two processes; the initiation of the formation of red cells is in the center of the marrow in zones of low circulation and always precedes the start of granulopoiesis. Shortly afterward, granulopoiesis begins along the border zone in the area most richly supplied with blood.

It is only for a brief period that there is this complete separation in the zones for the formation of red and white cells—the more central, less vascular zone of red cell formation and the peripheral, vascular zone for the formation of the white cells. Soon the two processes become more diffusely represented throughout the marrow, but always the fundamental relationship is maintained that the white cells develop around the patent sinuses and move toward their borders as they become more mature, while the red cells, on the other hand, develop in the areas of the closed sinuses, which, however, are connected with the patent sinuses through potential openings. In normal marrow the vascular and the avascular areas are thus close together instead of being peripheral and central in the marrow as a whole, as in the early stages. A peripheral, vascular, and thus granulopoietic area is maintained in the marrow of the adult rabbit, and hence remains the zone in which the status of leucogenesis can be studied in its simplest form.

The question of the myeloblast. Around the cells of level I, as shown in table I, have waged the discussions concerning cell potencies, so bewildering that Schridde (100) quotes Türk (101) as "saying resignedly" that "each one must have his own stem cell." Concerning the primitive reticular cell, primitive mesenchyme, whether in bone marrow or elsewhere, all observers might well meet on common ground as far as its power to give rise to the three strains of white blood cells—granulocytes, lymphocytes, and monocytes—is concerned, inasmuch as these cells are all mesenchymal in type. Beyond this point, the discussions center around the problem of the myeloblast, which has recently been reviewed by Downey (102), with a full bibliography. The cell later known as myeloblast was first described by H. F. Müller (103) from leucemic blood. The concept of the myeloblast announced by Naegeli

(92) in 1900 was, as Schilling has said, the beginning of light for this vexed question. The problem, if still at issue, is exactly where to place the power of trivalency; does it still exist in the primitive free cell, and have myeloblast, lymphoblast, and monoblast become determined toward the mature forms to which they correspond? For the discrimination of myeloblast and lymphoblast, there are three criteria: first, differences between the nuclei; second, the patterns of mitochondria, and third, the association with neighboring cells in the process of maturation (chart 1). Schridde (100) showed that the nuclei of the lymphoblasts have the denser membranes and the coarser chromatin network. For the cytoplasm there are the degree of basophilia and

TABLE 1.

LEVEL	BONE MARROW	LYMPH GLANDS	SPLEEN AND GENERAL CONNECTIVE TISSUES
I	Reticular cell	Reticular cell	Reticular cell
	Primitive free cell	Primitive free cell	Primitive free cell
	Myeloblast	Lymphoblast	Monoblast
II	Myelocyte A		
	Myelocyte B	Lymphocyte	Early monocyte
	Myelocyte C		Mature monocyte
	Metamyelocyte		
III	Leucocyte		

the number, size, and distribution of mitochondria. Differences in shape of mitochondria between myeloblasts and lymphoblasts have been described by Naegeli (92) and Schridde (100) in fixed films; differences in arrangement by Sabin *et al.* (93) Cunningham *et al.* (88), and by Simpson and Deming (104), from supravital studies with Janus green. How constant these patterns will prove and what their range of variation is must await further study.

In the study of leucemic blood, the actual diagnosis of myeloblastic leucemia is to be made, not because the morphological criteria, nuclear and cytoplasmic, just described are so clear and convincing as to admit of no differences in opinion, but rather because myeloblasts do not occur entirely alone, but rather in association with some young myelo-

cytes, those which show the onset of the next level, our type A, or myeloblasts with the so-called azurophilic progranulations. This was shown by Sabin *et al.* (93) in a case of myeloblastic leucemia. It has been confirmed with supravital studies by Simpson and Deming (104), who find in fact that in the blood of some cases of myeloblastic leucemia most of the cells may actually be the form of our myelocyte type A. It was also shown by Sabin *et al.* (93), confirmed by Richter (105), that it is the myelocyte type A that gives the meager but positive oxydase reaction, in contrast to the entirely negative reaction of myeloblasts. For the monoblast also the practical discrimination of the type is to be made in tissues through the actual association of the more differentiated types along with the immature. The value of the morphological criteria, nuclear and cytoplasmic, for discriminating immature blood cells is clear; these criteria, taken together with the fact that no one type in the constantly shifting stages of maturation, indicated on chart 1, occurs completely isolated from the adjacent stages, give data which are usually adequate for the diagnosis of the type of leucemia. The more theoretical question, perhaps the physiological problem, of the analysis of how far myeloblast, lymphoblast, and monoblast are already determined toward their mature strains must await the experimental evidence which will come as the nature of the chemical substances which control maturation is discovered.

Lymphocytes in bone marrow. With the simplification of our concepts of the structure of bone marrow, which began with the introduction of Naegeli's concept of the myeloblast, and with the realization that the primitive cells of the bone marrow are not identical with mature lymphocytes, the question of the occurrence of lymphocytes, not as scattered cells, but as developing locally in characteristic lymph follicles, becomes significant in relation to the question as to whether the primitive cells of the bone marrow are trivalent.

In 1902, Dominici (106) described rudimentary lymphoid follicles around the arteries of the marrow of a rabbit 15 to 20 days old; and since then the observation has been repeatedly made for human bone marrow. First, Longcope (107) reported finding follicles in human bone marrow in cases of typhoid fever; Hedinger (108) found germ centers in the bone marrow of a case of status lymphaticus; and Oehme (109) described typical lymphoid follicles with margins of mature

lymphocytes and centers of pale cells, lymphoblasts without the Schridde-Altmann granules (mitochondria), in a series of cases of pneumonia, rickets, and diphtheria in children. Askanazy (110) found follicles in 43 out of 126 cases, and viewed their occurrence not as a pathological, but rather as an occasional normal phenomenon. He found them not only in areas of the formation of red cells, but also in zones of active granulopoiesis. This work was extended by Fischer (111), who studied 61 cases ranging in age from 6 months to 84 years, and found follicles in 62 per cent. She gives many photographs both of single and of multiple follicles. The occurrence of lymph follicles has thus been amply confirmed (see also Aschenheim and Benjamin (112) and Weidenreich (113)), so that the possibility of the development of them in bone marrow may be considered as established, which brings evidence to bear toward the theory that the primitive cells of the marrow are trivalent.

In these studies it is striking how many of the observations have been made upon children, and especially in cases of rickets, but in the the surveys of Fischer the proportion of children with follicles was not greater than that of adults. The subject of the differences in the marrow of children and adults will be taken up later.

Monocytes in bone marrow. The monocyte does not occur in normal marrow in any large numbers, but monocytes can be stimulated to arise there. Doan and Sabin (85) have demonstrated this by massive injections of bovine tubercle bacilli in rabbits; the bone marrow becomes extensively involved with tubercles in the first three weeks. They made supravital studies of the marrow every day after the injection of tubercle bacilli; on the eighth day they found small clusters of the earliest monocyte that can be recognized, one with a single row of the characteristic neutral red bodies around the centropshere, corresponding to myelocyte A, and these young monocytes were intermingled with monoblasts and mature monocytes; at the same time the fat was beginning to disappear from the marrow. In succeeding days, the monocytes increased in numbers and in maturity and from them came typical epithelioid cells making tubercles.

To the fact that both lymphocytes and monocytes may arise in bone marrow it must be added that granulocytes may also arise in lymph glands (to be discussed later under "ectopic blood formation"). How-

ever, these facts do not alter the concept, which goes back to the work of Ehrlich, that normal adult bone marrow produces the granulocytes of the blood and the lymph glands the lymphocytes.

V. BONE MARROW AS AN ORGAN. It might be thought that the study of bone marrow as an organ should have begun with the discovery that blood cells arise there and with the pioneer work of Ehrlich; but following these early discoveries, attention was necessarily concentrated on the processes of maturation of red and white cells. Our knowledge of the evolution of blood cells has been gained more from the study of embryological stages before bone marrow functions, and far more from the study of peripheral blood, both in infections and in anemia and in leucemia, than from the study of normal bone marrow. As a result, the idea of studying bone marrow as an organ is relatively modern, and, inasmuch as the study of complex pathological marrow preceded a clear concept of its normal mechanism, a false idea has gained ground that marrow has a bewilderingly complex structure. Perhaps it is even simpler than subcutaneous tissue, for certainly fewer strains of cells are involved.

This modern phase of hematology, the period during which differential counts of the cells of the marrow have formed the basis for analysing the functional state of the organ, could not begin until certain fundamental points had been established. Three of these points have already been discussed: the clarification of the processes of maturation until there was a common basis of classification; the analysis of the nature of the vascular pattern of marrow, showing that collapsed capillaries favor erythrogenesis and dilated sinuses granulogenesis; the theory of the myeloblast as demonstrating that the output of the marrow in white cells in normally limited granulocytes, with lymphocytes and monocytes not involved. Beside these three factors, there is a fourth one of great importance, namely, an exact measure for the output of marrow. It has been found that there are various physiological outflowings of white cells from the blood into the tissues. Stockard and Papanicolaou (114) showed a marked migration of leucocytes into the vagina in guinea pigs at a certain stage of estrus; Isaacs and Danielian (115) have demonstrated an elimination of leucocytes into the saliva, which is probably indicative of a phenomenon in the entire digestive tract; Bunting and Huston (116) demonstrated a

similar migration of lymphocytes into the intestines. Beside these reactions, Barcroft *et al.* (58) found temporary storage of red cells in the spleen; and Goldscheider and Jacobs (117), discussed later, introduced the subject of redistribution phenomena of white cells in pathological reactions. All these observations make it clear that total and differential counts, giving the data for the number of cells in a cubic millimeter of blood, are alone insufficient to demonstrate an increased or decreased output from the marrow. An actual increase in immature forms must be known, the adequate criteria for which are the Arneth (98) "shift to the left" in the nuclear pattern for leucocytes and the reticulated count for the red cells.

The earlier of the modern studies of bone marrow involving differential counts to detect pathological changes were made by Rubinstein (118) in 1901, Price Jones (119), Wolownik (120), Bätge (121), Aschenheim and Benjamin (112) and Lossen (122). Rubinstein detected the shift to the less mature myelocytes in the marrow which is so constant a reaction under abnormal conditions. Lossen made counts of the total number of cells per cubic millimeter in children's marrow, finding that they ranged from 270,000 to 1,568,000 and that their number seemed, in a general way, to be dependent on the state of nutrition. The subject is one of extreme difficulty for numerical estimation, because of the uneven distribution of the zones of marrow between fat cells, but a general idea of the amount of marrow in terms of increased or decreased fat cells is of great importance; if Lossen's point of a decrease in the number of cells in the marrow of children under malnutrition be confirmed, it will have practical bearing. In this connection should be studied the results of Suzuki (123) on the blood-forming organs during inanition in guinea pigs. He found a lymphopenia corresponding to an atrophy of the lymphoid tissue generally correlated with a leucocytosis of the pseudoeosinophiles, due to a stimulus both of the marrow and to a granulopoiesis in lymph glands.

The more comprehensive recent studies on the subject of marrow have been made by Naegeli (82), Müller (51), Arneth (124), and especially by Schilling (125) and his students Bantz (126) and Yamamoto (127). Naegeli's studies led him to classify pathological changes of marrow into four types: erythroblastic, myelocytic, myeloblastic, and lymphatic. Schilling's classification involves five general types: 1,

ripe neutrophilic; 2, unripe neutrophilic; 3, ripe promyelocytic; 4, unripe promyelocytic, and 5, myeloblastic, to which are to be added the megaloblastic form of pernicious anemia, the lymphoid type of lymphatic leucemia, and the aplastic state, as special forms.

The studies of our group have been with normal bone marrow and with experimental studies that may be considered relatively normal (Cunningham and Doan, 73; Doan *et al.* 23; Doan and Zerfas, 128; Doan and Sabin, 85; Sabin and Doan, 129). The subject of bone marrow as an organ means the consideration of the entire marrow as a unit. It is an organ of no inconsiderable size, having been shown by Wetzel (130) to have a volume, in the adult human, of 1419 cu. cm., which is thirteen times that of the spleen, and almost equal to that of the liver.

Considering all of the marrow as a unit that is functioning in the formation of blood at any one time, that is, the entire marrow of rabbit or guinea pig or all the red in man, the whole structure will be found in a relatively uniform state under normal conditions. Since the marrow is carrying on two different processes, this statement means that the proportion of erythrogenesis to granulogenesis is relatively constant, as well as the proportions of the different levels of maturation within the two groups. This condition is not true when any deviation from the normal has brought about an extension of red marrow into the yellow, since there, first erythrogenesis, and later granulopoiesis will be found at earlier levels.

Comparative studies of blood and bone marrow for a group of five normal and two slightly abnormal rabbits were shown in three tables given by Sabin and Doan (129). A preliminary count of the blood was taken immediately before killing the animal, which was done by an injection of air into the veins. Normal marrow can only be analyzed with specific reference to the state of the blood of each animal; if other factors, peripheral redistribution and ectopic bone marrow, are ruled out, then the correspondence between blood and bone marrow will be found to be relatively exact.

For the surveys of the marrow, supravital preparations were made from the center of the shaft and from the epiphysis of each of the long bones from either side, and one preparation from a rib, making nine slides in all. From these preparations, from four to seven thousand

cells were counted by the two observers. There was no constant difference between erythropoiesis and granulopoiesis for shaft, epiphysis and rib, but because there are two processes occurring in different foci, it was found advisable to count about five thousand cells for comparable results.

In making the preparations, a small bit of the marrow was taken, avoiding the peripheral area of predominantly leucocytic function, and the areas between the fat cells, as well as some of the free cells along the border, were counted. For the zones between the fat cells, it was necessary to select areas that were relatively small, so that each cell could be identified; in avoiding the denser areas, it is probable that the number of the primitive forms of the white strain have been underestimated. The difficulties of the counting, due to the large number of cells and to the structural variation from two types of centers, make it hard to get surveys that are representative. The actual percentages cannot be regarded as even as close an approximation to the actual as blood counts, which themselves are likewise only approximations; nevertheless, the correspondence of the findings in the different animals to their own peripheral blood indicates that the results, if expressed in general terms, show a normal level for bone marrow as an organ.

The concept of the formation of white and red cells, given in the preceding pages, simplified such an experiment. This is true because all the stages of maturation of the red cells, the megaloblasts, erythroblasts, and normoblasts, as well as the preliminary endothelium, are readily discriminated in either fixed or supravital technique. For the fixed technique it is, of course, essential to be able to fix marrow before autolysis has taken place, and to use a fixing reagent without acid, for example, Helly's solution, in which neutral formalin is substituted for the acetic acid of the Zenker solution. For the white cells the classification given above makes the counts feasible, for the cells are considered in three groups, the agranular forms, the granular marrow cells, and leucocytes, with recognition of the range of variation in each group. From the data two points are to be analyzed, first, the normal proportion of erythroid to myeloid activity, and second, the proportions of the different stages within the two groups.

Ratio of erythropoiesis to granulopoiesis. For all seven animals the

total red counts of the peripheral blood were normal (5,500,000 for the rabbit), but in one, 19B, the fat was disappearing, indicating an extension of hematopoietic tissue, which involved an increased percentage (37) of red cells. With this exception, the ratio was about 70 per cent granulocytes to 25 per cent erythroid cells, with all other cells 5 per cent (table 2). Thus, for the normal rabbit, there are approximately three times as many myeloid cells as erythroid in marrow, as contrasted with a thousand times as many red cells as white in the peripheral blood. This reverse in the ratio must be correlated with the greater length of survival of the red cells in the blood stream. Schilling (125) with Banzler had an opportunity during the war to study the marrow

TABLE 2.

RABBIT NUMBER	PERIPHERAL BLOOD			BONE MARROW			NUMBER OF CELLS COUNTED
	White blood cells	Myeloid cells	Red blood cells	Total myeloid	Total erythroid	All other cells	
		total number		*per cent*	*per cent*	*per cent*	
1B	15,900	42% (6678)	6,360,000	72	26	2	4800
2B	12,600	40% (5040)	6,130,000	71	27	2	4200
3B	14,900	45% (6705)	5,800,000	71	19	10	7500
6B	11,900	48% (5712)	6,230,000	71	21	8	7300
7B	8,900	46% (4094)	5,230,000	68	28	4	6500
13B	12,200	83% (10,120)	5,610,000	63	27	10	4800
19B	14,800	71 (10,508)	6,050,000	59	37	4	5900

of ten healthy men, in whom counts of the blood had been made before death, and they found 44 per cent neutrophils plus eosinophiles (types not given) to 36 per cent normoblasts in the marrow. Doan and Zerfas (128) give the following myeloid-erythroid ratios for three accident cases: 80:5, 80:4, and 71:13; their myeloid cells include leucocytes, myelocytes, and myeloblasts. To establish the limits of variation of this ratio under normal conditions would be important.

Proportion of the different levels in granulocytes. The data concerning the myeloid elements alone are shown in table 3. Five of the rabbits had entirely normal blood, with total myeloid cells averaging 44 per cent, of which 36 per cent were pseudoeosinophiles (range 33 to 39 per cent), 7 per cent were basophiles (range 4 to 11 per cent), and 1 per

cent were eosinophiles (range 0 to 2 per cent). The other two animals were clinically normal, but were chosen for the study because of the increased percentages of granulocytes; R 13 B had 71 per cent pseudoeosinophiles, 10 per cent basophiles, and 2 per cent eosinophiles, making total myeloid cells 83 per cent; while for R 19 B the corresponding figures were 68, 2, and 1 with a total of 71 per cent. For the five normal rabbits, myelocytes C averaged 85 per cent, with myelocytes A and B, and myeloblasts in minimal numbers. Making due allowance for the limits of error in marrow counts, it is clear that normal marrow shows striking predominance of the myeloid cells near maturity.

TABLE 3.

Percentages of Types of Cells within the Myeloid Group in Bone Marrow.

RABBIT NUMBER	POLY-MORPHO-NUCLEARS	NEUTROPHILIC MYELOCYTES			MYELOCYTES		MYELO-BLASTS	MYELOID CELLS COUNTED
		C	B	A	Eosino-philic	Baso-philic		
1B	3.15	92.10	1.84	0.11	2.06	0.74	0	3,484
2B	7.34	79.38	8.35	0.43	2.37	1.57	0.56	2,988
3B	3.96	87.33	3.46	0.58	2.10	0.40	2.17	5,277
6B	1.93	90.87	3.16	0.36	1.22	0.84	1.62	5,215
7B	7.13	78.33	5.09	0.56	4.58	2.98	1.33	4,358
Average for five normal rabbits....	4.66	85.72	4.36	0.41	2.45	1.29	1.11	21,322
19B	19.99	54.43	19.29	0.76	2.36	2.95	0.22	3,514
13B	8.78	32.27	48.59	2.87	3.60	2.97	0.92	3,027

The condition of the marrow in the two animals in which the blood had indicated hyperactivity of the marrow in pseudoeosinophiles was striking. For R 19 B, the proportion of myelocytes C (54.43 per cent) had been diminished both by an increase in leucocytes up to 19.99 per cent, meaning the greater delivery into the blood, and by an increase in myelocytes B up to 19.29 per cent from the normal of 4.36 per cent. These cells were rather close to the C type, having more than half the full quota of granules. In the other rabbit, 13 B, the shift to immature forms was even more marked. Myelocytes C were 32.27 per cent, type B 48.59 per cent, and type A 2.87 per cent. In this animal myelocytes B were less mature, having on the average less than

half the full quota of granules. These two animals represent the condition of bone marrow corresponding to leucocytosis; it consists in an increased percentage of the early leucocytes (chart 1), together with an increased maturation of myelocytes from the earlier levels.

Reciprocal relationship of myelocytes C and early leucocytes. A point of significance in the study of the function of marrow follows from the predominance of the type C myelocyte, inasmuch as the delivery of cells from the marrow is made from this level. It was shown by Sabin *et al.* (99), in a study of human blood, that leucocytes enter the blood stream rhythmically with small hourly accessions and a larger daily rise in the afternoon. It was then demonstrated by Shaw (96), under the interesting title of "The diurnal tides of the leucocytes" that the afternoon rise is but a part of a diurnal rhythm, with a second rise about midnight. This rhythmic pattern in the delivery of cells was shown by Doan and Zerfas (128) to persist in cases with leucopenia or leucocytosis, the fluctuations being particularly striking during leucocytosis. They followed the Arneth pattern in seven cases, and demonstrated that these rhythms hourly and diurnal are actually due to an increased delivery of cells from the marrow.

The mechanism of the rhythmic delivery of cells from the marrow is in the reciprocal relationship between myelocytes C and early leucocytes. On chart 1, the legend *X-Y* on the line of granulations covers the group of cells which take part in this reciprocal relationship. These cells include myelocytes C and metamyelocytes on the left of the line, and the early leucocytes which Schilling (125), (131) has described under two names, *Jugendliche* and *Stabkern*, on the right. The classifying of the gradual changes of maturation (see chart 1) into fixed groups is just as difficult in the actual differential counting of cells as in the agreeing on a common nomenclature, and all figures from bone marrow must be studied with these difficulties in mind. In our counts the cells recorded as early leucocytes all showed active motility and hence are to be compared with Schilling's type *Stabkern* plus the few mature leucocytes from the circulating blood. The counts in table 3 show a range of from 3 to 7 per cent for early leucocytes for the first five animals, 8.78 per cent for rabbit 13 B, and 19.99 per cent in rabbit 19 B. Doan and Zerfas (128) give the following ratio of leucocytes to myelocytes in normal human bone marrow: 55:18,

10:55, and 13:54. The reciprocal relationship of myelocytes to early leucocytes is also shown by Schilling and Banzler for the ten normal human cases already mentioned. Their figures vary between the limits of myelocyte-leucocyte ratio of 34:56 and 47:44. Normal marrow has not been studied with reference to the variations in this ratio for a day, but it is probable that if marrow cells were taken by puncture in the early morning and just before the afternoon rise, the changes in the reciprocal relationship would be as clearly marked as the changes in peripheral blood, provided that enough cells were counted to offset the local nature of the phenomenon.

The approximate hourly delivery of cells to the blood suggests that the time for the maturation from myelocyte C to leucocyte might be expressed in units of hours or minutes. Doan, Zerfas, Warren and Ames (132) have demonstrated that the pouring out of new cells from the marrow occurs in from forty-five minutes to two and a half hours after sodium nucleinate, adenin and guanin nucleotides. It would be interesting to know how long the supply of myelocytes C might last, provided there was no immediate replacement from immature levels. The records of Doan and Sabin (85) of the study of the blood in rabbits with massive, intravenous doses of bovine tubercle bacilli give data that are suggestive in this connection. In the development of the leucopenia and anemia it took on an average eleven days for the marrow to reach the lowest level of red and white cells. In every case in which the animal survived the acute reaction, there was a disappearance of the tuberculosis from the marrow by lysis of the epithelioid cells; the onset of recovery in marrow function was first in platelets, then in the red cells, and it took about fifteen days for the red cells but only about eleven for the leucocytes to reach their original level, after which both passed above the original level, corresponding to a hyperplasia of the marrow before the final return to normal equilibrium. Thus, the time for the maturation of myelocytes A through to the myelocyte C may possibly be expressed in units of days.

In the counts of the marrow cells of the rabbit of Sabin and Doan (129), it is interesting to note that the eosinophilic myelocytes predominated over the basophilic, 2.6 to 1.8, while the reverse ratio was true for the blood, with one eosinophile to seven basophiles.

In making the correlation between the differential counts of Sabin and Doan (129) with other counts in the literature, it is necessary to relate the conventional nomenclature of the cells of level II, promyelocytes and myelocytes, heretofore used, with classification of myelocytes by the number of granules into types A, B and C. Pappenheim's term "*promyelocyte*" means cell with unripe granules. When neutrophilic granules (pseudoeosinophilic in the rabbit) first become visible, they are tiny and are quite acid or basophilic. They are spoken of as azur-progranulations. The changing chemistry of these granules, as they ripen, makes a difficult and uncertain basis for classification. Considering the matter first in supravital technique: neutral red is a chemical indicator with a range in pH from 6.8 to 8.0. The basophilic granulations are the most acid of all, and are a scarlet red; the earliest neutrophilic granules are red, but never quite as far to the acid end of the indicator as the true basophilic granules. As the cell matures, the chemical reaction shifts gradually toward the alkaline end with a more striking change detectable between myelocyte and early leucocyte, or between early leucocyte and leucocyte of the blood. The deeper stain of the granules of the myelocyte makes it a conspicuous cell in supravital preparations, and the same is true with early leucocytes when many of them have been called into the blood. The staining of the granules of the leucocytes of the Arneth range grows fainter and fainter as the cell matures, until in the non-motile phase there is no reaction at all. Thus the changing chemistry of the granules is a gradual phenomenon, extending from myelocyte type A to the senile leucocyte, with one sharp drop during the early leucocytes. The granules of the mature leucocytes do not represent an exact point in pH, but rather they function within a certain range. Whether the neutral red is a more or less sensitive indicator of this change than the methylene-blue-azur, or whether it registers exactly the same change, one cannot say; but in fixed technique the process is also gradual, with a marked change in the group of the myelocytes type C. That there is no exact point for the striking change accounts for the lack of a consistent and precise definition of "promyelocyte." There are three elements that make it impossible to define the exact limits between promyelocyte and myelocyte; first, the estimation of the color change may vary with the observer; second, more important is the fact that the color of the

granules in the fixed film varies markedly with the pH of the distilled water used in the technique; and third, probably different cells do not show the change at exactly the same point. It thus becomes necessary to estimate the definition of promyelocytes from the practical application in the counts in the literature. Schilling's definition is the most precise (125), and counts done in his laboratory (Yamamoto, 127) give for rabbit's marrow myelocytes 2 to 5 per cent, and promyelocytes

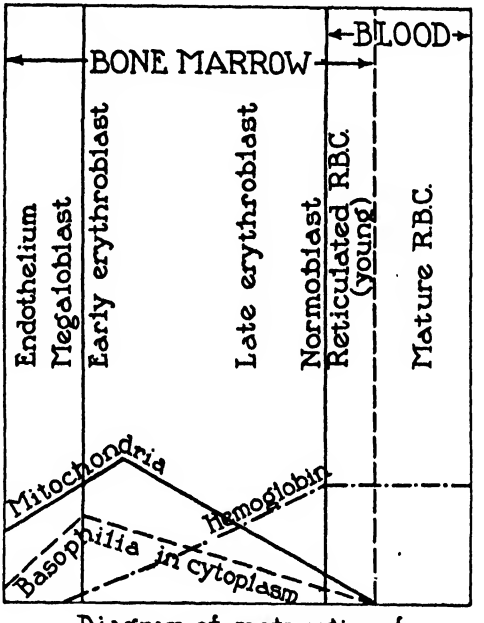

Diagram of maturation of red blood cells

CHART 2.

50 to 60 per cent. These figures indicate that promyelocyte may cover types A, B, and most of type C.

The changing chemistry of the granules is a fact, the physiological importance of which is at present probably underestimated; but as a basis of classification it fails to bring out the important fact that marrow functions from the mature levels. It has shown, it is true, that there is a shift to immature levels in pathological marrows, but it is not so sensitive an index of this change as the classification by the numbers of the granules.

Pattern of the red cells in bone marrow. A simplification of the study of red cells in marrow is involved in the work of Sabin (54) and Doan *et al.* (23), which led to the definition of the megaloblast as the earliest red cell, the direct derivative of endothelium. The maturation of the red cell is shown in chart 2. The critical points are at the megaloblast and normoblast. The latter is the cell of common size after the last division of the erythroblast. The mitochondria reach their maximum at the stage of the early erythroblast, the largest type in the development of the red cells, which was shown as figure 45 by Doan *et al.* (23).

The data for calculating the proportions of the stages of nucleated red cells in marrow were given by Sabin and Doan in their table 3 (129). As for the white cells, the major supply of nucleated red cells is at the more mature levels; the normoblasts make an average of 69 per cent of nucleated red cells; erythroblasts, early and late together, make 30 per cent, and the megaloblasts were found in only two animals, 0.01 and 0.04 per cent. The early erythroblasts run about 1 to 4 per cent.

The clumps of nucleated red cells in normal marrow are made up of late erythroblasts and normoblasts, and the rhythmic delivery of the red cells lies in the reciprocal relationship of normoblasts and reticulated red cells. Normal marrow does not draw upon endothelium and megaloblasts for its supply of red cells, and a simple anemia is represented in the marrow by an increase in the proportion of early erythroblasts. Only when a secondary anemia has become extreme is the pattern of the marrow thrown back to any extent to megaloblasts and endothelium. Sabin *et al.* (99) and Doan and Zerfas (128) have shown that the delivery of red cells from the marrow is rhythmic with a range of about 1,000,000 cells between the high and the low counts each day; as far as their observations have gone, the rhythm does not follow that of the white cells, with the marked rise in the afternoon, but rather the high points come both morning and afternoon.

Marrow in fetal stages and in infants. If bone marrow from a newborn rabbit be studied with the supravital technique, it will be seen that the proportion of erythroid to myeloid cells is practically the reverse of that of the adult, as estimated from films without actual counts. Such a marrow is most instructive for the early maturation stages of both red and white cells. As has been shown, these early

stages are present in such minimal numbers in adult marrow that they are difficult to demonstrate. In the newborn animal, on the other hand, the immature stages of both red and white strains exist in large proportion. In the red series, the early erythroblasts, many of them in the exceptionally large types of that stage, are easily found. For the white series also, myelocytes types A and B, and myeloblasts are in high proportions, with myelocytes C correspondingly reduced. This is excellent material for observing the small size and the different chemistry of the granules of the primitive myelocytes. In mature marrow, the numbers of the early myelocytes, with basophilic and eosinophilic granules, are too minimal to be observed, but on the other hand, they can be found easily in the marrow of the newborn.

Horwitz (133) found that in the human fetus of six months, the red cells predominated, making $\frac{3}{4}$ of the total; at eight months $\frac{9}{10}$, while at term she found the two strains equal. Lossen (122) found more erythroblasts in the newborn and during the first month of life than in the adult. Lateiner-Mayerhofer (134) found the marrow of the fetus predominantly agranular, with granulocytes running from 2 to 22 per cent before birth; in two babies that died at birth, the myelocytes were low, but there were 31 and 44 per cent leucocytes in the marrow, corresponding to the known leucocytosis. This decrease of leucocytes in the marrow lasted only a day, while the leucocytosis of the blood, 10,000 to 22,000, with 81 per cent neutrophilic cells, persisted three days, indicating redistribution phenomena. Her tables give a range of from 2 to 90 per cent of myeloid cells, which must be analyzed in terms of two different factors, namely, age given in months for the first year, and the varying diagnoses. Kramár and Heinsch (33) think that the marrow of the first year has 61 per cent granulocytes to 25 per cent lymphocytes, which is to be compared with the high percentage of lymphocytes, 27 to 44 per cent, the first eight years of life found by Doan and Zerfas (128). This point must be restudied with reference to the presence of lymph follicles in the marrow, and more especially in connection with the high relative percentage of lymphocytes to granulocytes in the blood of infants.

In earliest embryonic stages, only red cells are produced; during fetal stages, white cells are formed only in small numbers. It is clear, then, that there is a change in erythroid-myeloid ratio in the blood-

forming organs, from a beginning of 100 to 0 to an adult ratio in bone marrow around 1 to 3. In the pattern of both erythroid and myeloid elements, the marrow of the newborn represents a marked shift to immature forms, as compared with the adult pattern, or, stated the other way, during development the pattern of cells of bone marrow shifts toward the right, from a predominance of the immature types to a marked predominance of the mature stages. Thus it is easy to understand both why the cells of the blood of the newborn are, as a whole, shifted to the left, and how immature marrow cells, as, for example, myelocytes B, may appear in the blood with the same ease as the metamyelocytes in the adult.

Megalocaryocytes in bone marrow. Besides the specific forerunners of the blood cells, two other types of cells are found in bone marrow, megalocaryocytes and clasmatocytes. The multinuclear giant cells, the osteoclasts, which lie along the edges of the bone, are not here considered as cells of marrow. The megalocaryocytes have been related to the formation of platelets ever since the demonstration by Wright (135) that they give off masses of cytoplasm which fragment into platelets. This process was watched by Sabin (95) in megalocaryocytes which found their way into the peripheral blood.

In the normal rabbit these giant cells exist in small percentage, about 1 per cent in the marrow. It was shown by Frank (136) that the giant cells of the marrow decrease in proportion to a fall of platelets. Doan and Sabin (85) followed the fall in platelets which accompanies an anemia and leucopenia from an invasion of the marrow by tubercular tissue; in the subsequent recovery of the marrow, the platelets always started to rise first, then hemoglobin, then red cells, and finally the leucocytes. For every stage they killed a rabbit after counting the blood and found that degeneration and disappearance of megalocaryocytes paralleled the fall in platelets in the blood; and that, as the platelets rose, megalocaryocytes regenerated, first as small cells, with little cytoplasm in proportion to the nuclei. Finally, there were four or five in almost every field in contrast to the original number of one or two. Anselmino (137) found a decrease in megacaryocytes in the marrow associated with multiple hemorrhages in septic states.

Clasmatocytes. Clasmatocytes make also a small group of cells in normal marrow, running about 1 per cent in the rabbit. Doan and

Zerfas (128) found them in the same per cent in normal human marrow. They exist in two forms, scattered cells and the adventitial types of Marchand. They were shown by injections of trypan blue by Evans (138) and with India ink by Wislocki (139); their numbers become increased in the chronic reaction to particulate matter by a stimulus to endothelium (Doan *et al.*, 23). In the stage of the resolving tuberculosis of the marrow in rabbits, Doan and Sabin (85) found that the blood vessels were marked out by lines of adventitial clasmatocytes filled with acid-fast débris of the bacilli. In pernicious anemia, clasmatocytes increase in number, as shown by Doan (140), up to 10 per cent, and they become markedly phagocytic toward red blood cells, even engulfing the nucleated forms (Doan, 140; Peabody and Broun, 141).

Extramedullary formation of granulocytes and erythrocytes. If redistribution phenomena in the peripheral blood and an ectopic formation of bone marrow have been ruled out, the correlation between blood and bone marrow is quite exact. For example, in a series of tuberculous rabbits, of which repeated counts of the blood had been taken and charted, Doan and the writer made a correct estimation of the condition of the blood at the time of the death of the animal, from a study of the bone marrow alone in 66 out of 68 animals. Of the two in which the correlation was not made, one showed formation of red cells in the spleen, and so the regeneration of red cells was underestimated for the marrow, and in the other there had been too much autolysis of the marrow before fixation to make the study possible. It is well established that the formation of red cells or granulocytes may be easily shifted to the spleen, and this is thought to be explained by the fact that the formation of blood takes place normally in the spleen in late fetal stages. The formation of blood cells begins in the yolk sac; in the human embryo, it begins first in the body stalk and the more general connective tissues, and is at the start wholly of red cells. The process then passes first to the liver, and then to the spleen, before the bone marrow functions.

Beside this phenomenon, which may be regarded as a return to fetal conditions, as recently indicated by Ballin and Morse (142), there are two other types of extramedullary formation of myeloid and erythroid elements. The presence of granulocytic myelocytes, frequently

eosinophilic in type, diffusely scattered in connective tissues, is common in pathological conditions, both around chronic inflammatory areas and in the sepa of tumors (Weill, 143). Eosinophilic myelocytes are almost constantly present in the bronchial lymph glands of the pig, possibly correlated with the fact that the air passages practically always contain small, round worms. The question concerning the origin of such cells in the diffuse connective tissues is obscure. There are three possibilities: First, they might arise from the primitive cells along the blood vessels, in which case the stages of myeloblast, myelocytes A and B might occasionally be found. Second, they might arise from the division of myelocyte C from the bone marrow. Third, a number of recent observations tend toward the idea that cells now regarded as more differentiated types may give rise to granulocytes directly instead of by the process of maturation characteristic of bone marrow; for example, the view of Franco and Ferrata (144), and Ferrata (145) that the cell known as clasmatocyte, macrophage or reticulo-endothelial type, may elaborate specific granulations in its cytoplasm and become a granulocyte. They found such cells in the blood stream, containing either neutrophilic or eosinophilic granulations, and termed them "hemohistiocytes." These observations and interpretation have been confirmed by Richter (146) and by Jordan (147); but Doan and Sabin (148) have offered another explanation, namely, that neutrophilic and eosinophilic granules may be phagocytized from leucocytes and their débris, and that phagocytized fragments of red cells may be confused with true eosinophilic granules in fixed tissues.

Closely allied to Ferrata's view is the newer evidence from the method of tissue culture, which tends to show that the clasmatocytes and monocytes of the connective tissues and blood are the same cell under different phases of activity (Carrel and Ebeling, 149, 150; Lewis and Lewis, 151). Carrel and Rosenberger have shown by moving pictures of the living cells that both clasmatocytes and monocytes move by waving surface film, which is a type of motility quite different from that of leucocytes and lymphocytes. Carrel and Ebeling believe that one type can be changed into the other under the conditions of tissue culture through the control of the nourishment of the cells. Lewis and Lewis have added that the epithelioid cell also

belongs to the single strain of the phagocytic mononuclears, a view that has been confirmed by Gardner and Smith (152). Lewis (153) followed the transformation of granulocytes into macrophages in the living frog's lung. To these observations must be added the idea that mature lymphocytes of the tissues may elaborate the specific granulations and become leucocytes (Weidenreich, 154; Weill, 155, 156; Bloom, 157; Downey, 102). An analysis of the literature on this point is given by Downey. The occurrence of neutrophilic and eosinophilic myelocytes in the tissues abnormally must be studied in relation to the normal occurrence of histogenic basophiles. All these observations concern the relations of the mature or differentiated cells of the connective tissues to each other. On the functional side, Sabin, Doan and Cunningham (158), Cunningham *et al.* (88) and Sabin and Doan (159) regard the monocytes and the clasmatocytes as distinct strains and have shown that the epithelioid cell is derived from the monocyte. As an example of an important functional difference, Sabin and Doan have shown that the two cells react differently toward tubercle bacilli, inasmuch as this organism is to be found in the granular form in the clasmatocyte and in the rod form in the monocyte and epithelioid cell. The problem of the relationships of the cells of the connective tissues is very complex; it involves the consideration of both degeneration and de-differentiation *in vitro* as well as such possibilities *in vivo*. It is not possible at the present time to evaluate all the evidence concerning the reactions of the cells of the connective tissues to abnormal conditions; but whatever may prove to be the genetical relationships of the different types, it is probable that the differentiations of the cells of the connective tissues correspond to varying functional needs. These relationships of mature cells will only be analyzed through further chemical-biological studies, but this particular problem is not involved in the simpler processes of maturation of leucocytes in normal bone marrow. Ono (160) has brought about the extramedullary formation of both granulocytes and normoblasts by means of injections of red blood corpuscles from which the hemoglobin had been removed with distilled water. The condition, discussed later, involved a marked stimulus to erythrogenesis in the marrow.

Beside this diffuse reaction of granulocytic types in the connective tissues, ectopic bone marrow as a definite nodule has been frequently

observed. Recent reports (Brannon, 161; Saleeby, 162; Lang, 163) give the literature. Such nodules have a definite vascular pattern; they are frequently predominantly of red cells and have a pattern of definite collapsed capillaries, like that of gelatinous bone marrow. Bone marrow occurs also in cartilage usually wholly hyaline; it has been produced experimentally by slowing the blood flow (Sacerdotti and Frattin, 164; Maximow, 165).

Vasomotor reactions. With the concept in mind that bone marrow as an organ is the place where two types of cells are made, that it holds a large store of each of the two strains almost ready for delivery, and that its store of immature forms is small in numbers, but of exceedingly high potentiality toward multiplication, growth, and maturation, the question of the mechanism to maintain such a normal structure becomes the major problem of hematology.

The mechanism involves both vasomotor and chemical regulation. The newer work on the vascular system (Doan *et al.*, 23) shows that whatever controls the proportion of collapsed capillaries to open sinuses is an important factor in the regulation of the proportion of red cells to myeloid elements. Thus the problem of vasomotor control of marrow is of great significance. It is clear that there are dynamic factors due to the fact that the marrow is in a closed box; second, that vasomotor control by the action of nerves on the muscle of the arteries is important, and that all the factors in control of the closing of capillaries discussed by Krogh (5), Hooker (74), (75), Richards (77) and Rich (76) are met here with the further complication of the special dynamic actors. Thus, while increased and decreased blood flow through the vasomotor nerves of the arteries plays a part, it is not clear whether the collapsing of the capillaries is regulated in part by decreased blood flow and in part by a local chemical stimulus applied to endothelium. In marrow, the collapsed capillary gives areas of prolonged low oxygen tension. Beside the important factor that regulates the proportion of open to collapsed capillaries in active marrow, the mechanism that controls the long continued low blood supply in yellow marrow is also significant.

When the pattern of levels within each group is considered, two different processes must be analyzed: first, the mechanism of the delivery of cells to the circulation, and second, the mechanism of

maturation. Concerning the mechanism for the delivery of cells into the circulation, the question of how great a part vasomotor influences play is uncertain. Vasomotor phenomena with reference to blood have been studied by Ribadeau-Dumas and Roussy (166), Glaser (167), (168), (169), Glaser and Buschmann (170), Müller (171) and Yamamoto (127). The response of the marrow to the nerve stimuli has not been sufficiently controlled with reference to the normal rhythms of the blood cells and to the fact that the range of normal variation in a day is 100 per cent, as is shown when counts are taken every fifteen minutes for several hours (Sabin *et al.*, 99). Glaser and Buschmann (172) found a variation of 53 per cent in a day, of 76 per cent between different days, and finally of 90 per cent. The leucocytosis following adrenalin is lymphocytic rather than myeloid (Doan and Zerfas, 128). Ribadeau-Dumas and Roussy cut the nerves of the hind leg in a rabbit and found less output in response to collargol; Yamamoto cut the nerves of the leg in eight rabbits; after four days he gave injections of streptococci and studied the marrow three days later. He found a marked increase in the cells of the marrow and discovered that they were more mature in the leg without nerves. He concluded that while maturation is a chemical phenomenon, the calling of cells from the marrow is vasomotor. It is not likely, however, that such a separation of vasomotor and chemical factors can be made. For the admission of cells into the circulation two structures are involved: the endothelial walls of the sinuses and the blood cells themselves. A stretching of the wall of the sinusoids favors the entering of leucocytes between endothelial cells. This can be seen by watching the living vascular membranes from a chick of the sixth and seventh days of incubation; the young granulocytes exist there, as in adult marrow, not singly but in groups at the same stage of maturation; such a group of granulocytes can be seen to move *en masse* against the wall of the capillary until the wall is bent inward; when the stretching reaches a certain point a leucocyte close to the wall flows in between two endothelial cells and then the rest all follow in rapid succession. The same process of stretching of the walls of capillaries was shown in the early studies of inflammatory tissues to let the leucocytes out of the vessels.

For the red cells, it is easy to see that a constriction or shortening of

the endothelial wall just at the point where a narrow neck connects the patent sinusoid with a hematogenic capillary distended with developing cells, would favor the opening of the capillaries. The mechanism of such a local lengthening and shortening of endothelial walls, whether passive or related to increased or decreased blood flow, or to local chemical stimuli for endothelium, has not been analyzed. However, the nice adjustment which results in the normal admission of only those cells that are functionally mature must also involve chemical stimuli that affect the blood cells directly only when they are mature. For the white cells such chemotactic factors have long been known.

Chemical factors. A leucocytosis involves both an increased delivery of cells from marrow and an increased maturation of myelocytes B and finally of type A; these two reactions are to be expressed as the result of two different factors, a chemotactic factor (C) to account for the increased delivery of cells and a growth-stimulating or maturation factor (M) for the shift to the less mature cells in the marrow pattern. Muir (173) in 1898 analyzed leucocytosis as depending on two processes but thought of them as due to the same substance.

If, on the other hand, inactivated typhoid bacilli be administered to a rabbit and the blood be followed through the stage of the leucocytosis, the marrow will finally be found entirely depleted of both myelocytes C and B and reduced to the low level of myelocytes A and myeloblasts (Doan *et al.*, 23). Such a marrow corresponds to a state of leucopenia, and the experiment can be expressed as representing a condition due to a chemotactic factor minus a maturation element.

This concept of leucocytosis and leucopenia postulates the infecting organism as introducing both a chemotactic and a maturation factor when a leucocytosis results and a chemotactic factor alone when a leucopenia follows a temporary leucocytosis. The question may be raised as to whether a leucopenia may not be due rather to a depressant factor than to the absence of a stimulating substance. In the experiments cited with inactivated typhoid bacilli, the cells in the marrow showed no toxic effect and the marrow readily regenerates after the experiment. In typhoid fever itself a totally different element is introduced as shown by Quincke and Stühlen (45), Fraenkel (46), (47) and Hartwich (48), because the marrow is frequently infected locally, with abscesses, necrosis and hemorrhage.

Knowledge of chemotactic reactions on blood cells goes back to Cohnheim and to Ehrlich. The existence of such substances in bacterial proteins was shown by Buchner (174) who obtained albuminates from the Friedländer bacillus and showed that they produce pus. Roemer (175), (176), followed the development of a leucocytosis produced with the bacterial protein of Buchner and with Koch's tuberculin as chemotactic factors. The first suggestion of such a chemotactic factor produced by the body itself was by Horbaczewski (177) who thought that digestion leucocytosis was correlated with an increase in uric acid in the blood. It is now known, however, that digestion leucocytosis does not exist, for the afternoon rise in leucocytes has been shown to take place whether food is taken or not (99).

Of the many substances that have been shown to call leucocytes from marrow, nucleic acid is most likely to be a part of the normal mechanism. Löwit (178) found a leucopenia followed by a leucocytosis after a large number of substances, bacterial proteins, hemialbumose, peptone, pepsin, and nucleic acid and nuclein; he also included from the literature blood extracts, curare, uric acid and sodium urate. He thought that the leucopenia was due to a destruction of the leucocytes and that the leucocytosis was the reaction of the marrow to replace them. Thus his theory was that leucolysis and leucocytosis were cause and effect. Goldscheider and Jacobs (117) repeated the experiments and regarded the phenomenon as due to chemotaxis. They brought forward the view that the leucopenia which is the initial reaction to hemialbumoses, nucleic acid, tuberculin, bacterial proteins, and organ extracts is due especially to a lodging of leucocytes in the lung. Thus they introduced the important idea that the analysis of a chemotactic action on the marrow depends on the understanding of peripheral redistribution phenomena in order to distinguish exactly how much of an observed effect on the blood cells is due to action on bone marrow.

The most recent contribution on this subject is that of Doan, Zerfas, Warren and Ames (132) in a study of the effect of large doses of nucleic acid. They have demonstrated that the phenomenon of peripheral redistribution of blood cells can only be studied in the living animal. With the group of four workers they made synchronous repeated counts of the blood from a peripheral vein, the heart, the liver, and the

spleen. The animal was kept anesthetized for hours under sodium barbital. From the liver and the spleen they cut tiny bits of the tissue for histological control at the time of each count. In the study of the leucopenic phase, the counts of blood from three places, peripheral vein, heart, and liver, ran parallel, all showing the leucopenia, while there was steady increase in the percentage of granulocytes in the spleen, correlated with an increase in the size of this organ, as determined by the oncometer. The piling up of granulocytes in the spleen is also shown in their photographs.

That the spleen was the only organ involved in the leucopenia was demonstrated by the complete elimination of this phenomenon after splenectomy. After making a long series of counts of the blood from the liver, they repeatedly showed that the only striking change in the white cells in that organ came at the moment of the death of the animal; moreover, the change, starting as soon as the punctures could be made after death, consisted only in an increase in the total number of white cells per cubic millimeter with no change whatever in the proportions of the different types of white cells. It is thus clear that all studies of redistribution phenomena must be made on the living animal with both total and differential counts.

On the other hand, their studies of the bone marrow showed clearly a chemotactic effect of the nucleic acid, with the massing of leucocytes around the patent sinusoids, a marked diapedesis into the vessels, and the vacant areas of the marrow from which the granulocytes had been drawn. The increase of the granulocytes in the blood stream was temporarily masked by their storage in the spleen, with the resulting leucopenia. They then found that the granulocytes could be called from the marrow by the split products of nucleic acid, adenin, and guanin nucleotide, and that after these substances the leucocytes were not withdrawn from the circulation by the spleen so that there was a direct leucocytosis without the temporary leucopenia. It is thus likely that nucleic acid and its derivatives are important physiological factors in the chemotactic reaction, and that the showers of non-motile leucocytes in the circulating blood may give a rhythmic discharge of such products into the circulation. It will be interesting if the rhythmic extrusion of the nuclei of normoblasts plays a rôle in this phenomenon.

Thus there are many observations of chemotactic substances and quite definite evidence concerning their physiological mechanism. Of maturation factors there is but meager knowledge. In this connection it is well to make clear that three processes are involved in the maturing of the marrow cells: first, cell division; second, the growth of cells between the cycles of division; and third, the varying factors in the cytoplasm which are represented on charts 1 and 2, such as the elaboration of specific granules, or hemoglobin, and the decrease of other substances built up in the earlier stages. To these must be added nuclear changes such as the loss of nucleoli in the more mature stages and the increase in the amount of chromatin. The physiological maturation factors for white cells are not known, but we have postulated that those bacteria that produce a sustained leucocytosis introduce such a factor, for they produce an increased division, growth, and maturing of the less mature myelocytes in the marrow far beyond the normal amount. Bacon, Novy and Eppler (179), while not discriminating between chemotactic and maturation factors, consider that even in infections the stimulus to an increased activity of the marrow comes from altered body proteins. They have studied dehydration phenomena from the injection of hypertonic solutions and postulate that the resulting fever and leucocytosis are like the reactions of infections and due to changes in the body proteins from the hydration. Thus they consider that the mechanism of the leucocytosis in an infection is due to an increase of the substances in the body that afford the normal stimulus, rather than to some substance from the bacteria. The relationship of the degree of the leucocytosis to the resistance of the animal in infections has been repeatedly confirmed since Metchnikoff; and thus, as long as none of the substances involved in these reactions is known, variations in response of the animal must be studied in terms of the amount of the infection and possibly differences in the power of the hematopoietic tissues to respond.

The only knowledge of maturation factors concerns red cells. The recent application of the liver diet to pernicious anemia (Minot and Murphy, 68, 69) and the isolation from liver of the specific substance (Cohn *et al.*, 180) involve the discovery of a maturation factor. It has long been known that megaloblasts were to be found in increased numbers in the marrow in pernicious anemia, but only the recent studies

with material taken at biopsy (Zadek, 181; Peabody, 34) have made it clear that in the height of a relapse the bone marrow of pernicious anemia may be defined as exhibiting a condition in which the maturation of the red cells has been arrested in the megaloblastic phase. In terms of the normal pattern of erythrogenesis, the marrow has been changed from the normal predominance of normoblasts, 69 per cent, to a much greater predominance of megaloblasts. However, the condition is not only an extreme shift to the left in erythrogenesis in the marrow, but there is also disorder of maturation with the disappearance of the mechanism for the reduction of the red cells to a standard size. The second line on chart 2 has to be erased to express the condition. In the peripheral blood the red cells normally continue to grow smaller by fragmentation, a process markedly increased in secondary anemia (Rous and Robertson, 182, 183; Doan and Sabin, 148), but cells larger than normal can only come from disorganized maturation. Minot and Murphy (68), (69) have shown that by the liver diet the normal mechanism of maturation is restored by the speedy appearance in the peripheral blood of reticulated red cells of normal size.

The recent discovery by Welo and Baudisch (184), (185) of "active iron," which is a ferromagnetic cubic form of iron oxide reopens the whole subject of the effect of iron on erythrogenesis. It has already been used successfully in the treatment of anemias of children by Moldawsky (186) and must be studied experimentally in its action on bone marrow. The work of Ono (160) is likewise important. He took blood, washed the corpuscles repeatedly with salt solution to free them from serum, pipetted off the leucocytes and then shook the red corpuscles with distilled water until completely hemolyzed. He found that the corpuscles freed of the iron content were a marked stimulus for erythropoiesis in rabbits when given in medium doses, 2 to 3 cc. per kilo for autologous corpuscles, 3 to 8 cc. for isologous and 8 to 30 cc. per kilo for heterologous cells. These injections were repeated every day, or every other day until a certain amount had been given. Small doses were ineffective and large doses toxic. The medium doses corresponded to the estimated physiological destruction of red cells per day. There was an increase in red cells, with the reticulated forms, reaching a maximum in from 2 to 7 hours, with a fall to the original level and a subsequent rise in 2 to 3 days which was sustained for a few

weeks. In the tables the change was about a million cells during the sustained rise. Controls were made with hematin and extracts from lymph glands, muscle, and kidney without the specific effect on erythrogenesis.

From the preceding pages the following points concerning the structure of bone marrow as an organ can be summarized. There is a normal proportion of erythroid to myeloid activity for the adult, which is the reverse of that at the time of birth. There is a normal pattern of maturation in each group, erythroid and myeloid. For each group the major supply of cells is at a stage near maturity, with the more primitive stages represented by minimal numbers. The delivery of cells to the circulation is from a reciprocal relationship between the late stages of marrow cells and the early phases of the blood cells. The patterns of marrow and the rhythmic delivery of cells into the circulation depend on the fact that maturation and cell division in marrow occur in rhythmic, orderly cycles. Blood formation begins in the earliest embryological stages with red cells alone; white cells are in minimal numbers in fetal stages; the pattern of marrow at birth shows a predominance of the early phases of each strain the reverse of the adult pattern, and the maturing of bone marrow involves a gradual shifting of the entire pattern to the stage of a predominance of the more mature marrow cells. Most pathological changes in marrow involve a shifting back toward the left from the adult pattern. Whenever the function of bone marrow has been depressed in both red and white strains, the onset of recovery is first in erythrogenesis, the red cell being of more fundamental importance to the organism. Normal maturation involves giving to the blood both red and white cells of a uniform size, since neither definitive red cells nor granulocytes divide, and in a relatively uniform state of maturity.

The earlier discussions concerning monophyletism, dualism, and polyphyletism were concentrated on the problem of exactly where in the cells of level I, table 1, came the irreversible determination into one of the three strains, the granulocyte, lymphocyte, or monocyte. The modern aspect of this problem can be stated as follows: In bone marrow the normal mechanism is adapted to the production of three strains of cells, red cells, granulocytes, and megacaryocytes for the platelets. Collapsed capillaries make zones of low oxygen

tension which favor red cells; widely dilated sinuses give abundant and slow blood flow which brings the maturation factors in the right concentration for granulocytes between these vessels. In lymph glands the abundant and slowly flowing lymph of the lymphatic sinuses brings the maturation factors in the right concentration to produce lymphocytes. Monocytes are produced in small numbers in the diffuse connective tissues. These are the normal reactions, but bone marrow may be thrown over to the production of almost no white cells but lymphocytes in lymphatic leucemia, or almost no cells but monocytes in massive tuberculosis of the organ. Granulocytes or monocytes may be formed in lymph glands, and granulocytes, lymphocytes, or monocytes may be produced in the connective tissues. The modern aspect of the problem involves a shift in the emphasis from the morphological question to the investigation of the chemical maturation factors which determine these three strains of cells. These chemical factors are to be sought through the chemical analysis of bacteria known to affect blood cells, in certain organ extracts, as well as in specific dietary factors, some of which may be vitamines (Whipple *et al.*, 66, 67; Minot and Murphy, 68, 69; Drew and Mottram, 187; Koessler and Maurer, 188).

Pathological conditions in bone marrow can be divided into three groups. First, the conditions of simple leucocytosis and leucopenia together with secondary anemia and aplasia, in which the normal pattern of marrow has been shifted toward the less mature stages, with or without increased maturation from the immature cells. This condition involves no deviation from the normal rhythmic division and maturation. Second, states like pernicious anemia and leucemia, in which the pattern of the marrow has also shifted toward the immature states, even to the most immature, myeloblasts and megaloblasts, but with complete disorder in cell division and in maturation, so that immature cells of all sizes and stages of development come to the blood stream. Third, conditions in which there has been invasion of the marrow by infections or with new growth of tissues involving such phenomena as replacement, necrosis and hemorrhage, rather than disorders involving only the maturation patterns.

With such an analysis, it is evident that a start has already been made in a new type of hematological research in which the goal is to

find the chemical substances which affect the endothelium of sinusoids and capillaries, the primitive mesenchyme and the marrow cells, both red and white, and thus regulate the normal mechanism of bone marrow as an organ. Such knowledge would make a basis for unraveling the conditions which underlie abnormal changes in the mechanism.

BIBLIOGRAPHY.

I. The Vascular System.

(1) Woollard, H. H. and G. B. Wislocki. Johns Hopkins Hosp. Bull., 1923, xxxiv, 418.
(2) Doan, C. A. Johns Hopkins Hosp. Bull., 1922, xxxiii, 222.
(3) Doan, C. A. Carnegie Institution of Washington, Pub. no. 277. Contrib. Embryol., 1922, xiv, 27.
(4) Krogh, A. Journ. Physiol., 1918–1919, lii, 457.
(5) Krogh, A. The anatomy and physiology of capillaries. New Haven, Yale University Press, 1922.
(6) Minot, C. S. Proc. Boston Soc. Nat. Hist., 1901, xxix, 185.
(7) Bizzozero, G. and A. A. Torre. Untersuchungen zur Naturlehre des Menschen und der Thiere. Giessen, Moleschott, 1881, xii, 626.
(8) Denys, J. La cellule, 1887, iv, 200.
(9) Van der Stricht, O. Arch. de Biol., 1892, xii, 199.
(10) Bizzozero, G. Topographia Italiana, 1869.
(11) Neumann, E. Arch. d. Heilkunde, 1869, x, 68.
(12) Neumann, E. Centralbl. f. d. med. Wissensch., 1868, vi, 689.
(13) Bizzozero, G. Centralbl. f. d. med. Wissensch., 1868, vi, 885.
(14) Neumann, E. Arch. d. Heilkunde, 1869, x, 68.
(15) Von Recklinghausen, F. D. Die Lymphgefässe und ihre Beziehung zum Bindegewebe. Berlin, A. Hirschwald, 1862.
(16) Hoyer. Arch. f. Anat. u. Physiol. u. wissensch. Med., 1865, p. 204.
(17) Hoyer, H. Centralbl. f. d. med. Wissensch., 1869, vii, 244, 257.
(18) Langer, K. Denkschr. k. k. Akad. d. Wiss. Wien. Math. Naturw. Kl., 1877, xxxvii, 217.
(19) Rindfleisch, G. E. Arch. f. mikr. Anat., 1879–1880, xvii, 1, 21.
(20) Muir, R. Journ. Path. and Bact., 1894, ii, 354.
(21) Maximow, A. Beitr. z. path. Anat. u. allg. Path., 1907, xli, 122.
(22) Drinker, C. K., K. R. Drinker and C. C. Lund. Amer. Journ. Physiol., 1922, lxii, 1.
(23) Doan, C. A., R. S. Cunningham and F. R. Sabin. Carnegie Inst. of Washington, Pub. no. 361, Contributions to Embryology, 1925, xvi, 163.
(24) Bunting, C. H. Journ. Exper. Med., 1906, viii, 625.
(25) Firket, J. Compt. rend. Soc. biol., 1921, lxxxv, 727.
(26) Jordan, H. E. and J. P. Baker. Anat. Rec., 1927, xxxv, 161.
(27) Mollier, S. Arch. f. mikr. Anat., 1910–1911, lxxvi, 608.

II. Distribution of Red, Fatty and Gelatinous Marrow.

(28) Piney, A. Brit. Med. Journ., 1922, ii, 792.
(29) Ghedini, G. Wien. klin. Wochenschr., 1910, xxiii, 1840.
(30) Ghedini, G. Wien. klin. Wochenschr., 1911, xxiv, 284.
(31) Morris, L. M. and E. H. Falconer. Arch. Int. Med., 1922, xxx, 485.
(32) Caronia, G. La Pediatria, 1922, xxx, 607.
(33) Kramár, E. and V. Hensch. Monatsch. f. Kinderheilk., 1925, xxx, 440.
(34) Peabody, F. W. Amer. Journ. Path., 1927, iii, 179.
(35) Seyfarth, C. Deutsch. med. Wochenschr., 1923, xlix, 180.
(36) Yamamoto, T. Virchow's Arch. path. Anat., 1925, cclviii, 62.
(36a) Meltzer, S. J. Amer. Journ. Physiol., 1916, xl, 126.
(37) Bloor, W. R. Journ. Biol. Chem., 1925, lxiii, 1.
(38) Bichat, X. Anatomie Générale. Pt. II, T. iii, p. 116. Paris. Brosson, Gabon et Cie., 1801.
(39) Jackson, C. M. Arch. f. Anat. u. Physiol., Anat. Abt., 1904, 33.
(40) Neumann, E. Centralbl. f. d. med. Wissensch., 1882, xx, 321.
(41) Muir, R. Journ. Path. and Bact., 1901, vii, 161.
(42) Dickson, W. E. C. The bone-marrow. A cytological study. New York, Bombay and Calcutta, Longmans Green & Co., 1908.
(43) Stefko, W. H. Ergebn. d. allg. Path. u. path. Anat., 1927, xxii, Abt. I, 739.
(44) Johnstone, E. M. Arch. Surgery, 1922, v, 159.
(45) Quincke, H. and A. Stühlen. Berl. klin. Wochenschr., 1894, xxxi, 351.
(46) Fraenkel, E. Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1903, xi, 1.
(47) Fraenkel, E. Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1903, xii, 419.
(48) Müller, E. F. Zeitschr. f. Hyg. u. Infektionskr., 1921, xciii, 223.
(49) Hartwich, A. Frankfurter Zeitschr. f. Path., 1921–1922, xxvi, 227.
(50) Hartwich, A. Virchow's Arch. path. Anat., 1921, ccxxxiii, 425.
(51) Müller, E. F. Virchow's Arch. f. path. Anat., 1923, ccxlvi, 49.

III. Formation of Red Blood Cells in Marrow.

(52) Bizzozero, G. Arch. f. mikr. Anat., 1890, xxxv, 424.
(53) Danchakoff, V. Anat. Hefte, I Abth., 1908, xxxvii, 471.
(54) Sabin, F. R. Carnegie Inst. of Washington, Pub. no. 272. Contributions to Embryology, 1920, ix, 213.
(55) Bert, P. Compt. rend. de l'Acad. d. Sci., 1882, xciv, 805.
(56) Viault, F. Compt. rend. d. l'Acad. d. Sci., 1890, cxi, 917.
(57) Douglas, C. G., J. S. Haldane, Y. Henderson and E. C. Schneider. Phil. Trans. Roy. Soc. London, Ser. B., 1912–13, cciii, 185.
(58) Barcroft, J., C. A. Binger, A. V. Bock, J. H. Doggart, H. S. Forbes, G. Harrop, J. C. Meakins and A. C. Redfield. Phil. Trans. Roy. Soc. London, Ser. B., 1922–23, ccxi, 351.
(59) Brill, N. E. Med. Record, 1913, lxxxiii, 776.

(60) DALLWIG, H. C., A. C. KOLLS AND A. S. LOEVENHART. Amer. Journ. Physiol., 1915–1916, xxxix, 77.
(61) JORDAN, H. E. AND C. C. SPEIDEL. Proc. Soc. Exper. Biol. and Med., 1923–1924, xxi, 399.
(62) CAMPBELL, J. A. Journ. Physiol., 1927, lxiii, 325.
(63) CAMPBELL, J. A. Journ. Physiol., 1926–27, lxii, 211.
(64) MULLER, G. L. Amer. Journ. Physiol., 1927, lxxxii, 269.
(65) SUGIYAMA, S. Carnegie Inst. of Washington, Pub. no. 363. Contributions to Embryology, 1926, xviii, 121.
(66) WHIPPLE, G. H., F. S. ROBSCHEIT AND C. W. HOOPER. Amer. Journ. Physiol., 1920, liii, 236.
(67) ROBSCHEIT-ROBBINS, F. S. AND G. H. WHIPPLE. Amer. Journ. Physiol., 1925, lxxii, 408, 431.
(68) MURPHY, W. P. AND G. R. MINOT. Boston Med. and Surg. Journ., 1926, cxcv, 410.
(69) MINOT, G. R. AND W. P. MURPHY. Journ. Amer. Med. Assoc., 1926, lxxxvii, 470.
(70) MUIR, R. Trans. Path. Soc., London, 1902, liii, part III, 379.
(71) SELLING, L. Zeigler's Beiträge z. path. Anat., 1911, li, 576.
(72) SELLING, L. Johns Hopkins Hosp. Reports, 1916, xvii, 83.
(73) CUNNINGHAM, R. S. AND C. A. DOAN. Proc. Soc. Exper. Biol. and Med., 1922–1923, xx, 262.
(74) HOOKER, D. R. Amer. Journ. Physiol., 1920–21, liv, 30.
(75) HOOKER, D. R. Physiological Reviews, 1921, 1, 112.
(76) RICH, A. R. Journ. Exper. Med., 1921, xxxiii, 287.
(77) RICHARDS, A. N. Amer. Journ. Med. Sci., 1922, clxiii, 1.
(78) RICHARDS, A. N. AND C. F. SCHMIDT. Amer. Journ. Physiol., 1922, lix, 489.
(79) BAYNE-JONES, S. Amer. Journ. Anat., 1917, xxi, 449.
(80) KEY, J. A. Arch. Int. Med., 1921, xxviii, 511.
(81) PEABODY, F. W. Amer. Journ. Path., 1926, ii, 487.
(82) NAEGELI, O. Blutkrankheiten und Blutdiagnostik. Ed. 4, Berlin. Julius Springer. 1923.
(83) MULLER, G. L. Journ. Exper. Med., 1926, xliii, 533.
(84) MULLER, G. L. Journ. Exper. Med., 1927, xlv, 399.
(85) DOAN, C. A. AND F. R. SABIN. Journ. Exper. Med., 1927, xlvi, 315.
(86) KOOLMANN, M. TEN D. Klin. Wochenschr., 1926, v, 709.

IV. FORMATION OF WHITE BLOOD CELLS IN MARROW.

(87) MALLORY, F. B. AND F. PARKER, JR. Amer. Journ. Pathol., 1927, iii, 515.
(88) CUNNINGHAM, R. S., F. R. SABIN AND C. A. DOAN. Carnegie Inst. of Washington, Pub. no. 361, Contrib. Embryol., 1925, xvi, 227.
(89) ORSÓS, F. Beitr. z. Path. Anat., 1926–27, lxxvi, 36.
(90) LANG, F. J. Arch. Path. and Lab. Med., 1926, i, 41.

(91) KUBIE, L. S. Journ. Exper. Med., 1927, xlvi, 615.
(92) NAEGELI, O. Deutschr. med. Wochenschr., 1900, xxvi, 287.
(93) SABIN, F. R., C. R. AUSTRIAN, R. S. CUNNINGHAM AND C. A. DOAN. Journ. Exper. Med., 1924, xl, 845.
(94) PAPPENHEIM, A. Die Zellen der leukämischen Myelose. 2 vols., Jena, Gustav Fischer, 1914.
(95) SABIN, F. R. Johns Hopkins Hosp. Bull., 1923, xxxiv, 277.
(96) SHAW, A. F. B. Journ. Path. and Bact., 1927, xxx, 1.
(97) STETSON, R. P. Arch. Int. Med., 1927, xl, 488.
(98) ARNETH, J. Die qualitative Blutlehre. 3 vols., 1920–1925, Leipzig, W. Klinkhardt and Münster Stenderhoff.
(99) SABIN, F. R., R. S. CUNNINGHAM, C. A. DOAN AND J. A. KINDWALL. Johns Hopkins Hosp. Bull., 1925, xxxvii, 14.
(100) SCHRIDDE, H. Beitr. path. Anat., 1907, xli, 223.
(101) TÜRK, W. Vorlesungen über klinische Hämatologie, 2 vols. in 3 Pts., Wien und Leipzig, 1904–1912, Wilhelm Braumüller, (see p. 152, vol. ii, Pt. 1).
(102) DOWNEY, H. Folia Haemat., Teil I, 1927, xxxiv, 65–89 and 145–187.
(103) MÜLLER, H. F. Deutsch. Arch. f. klin. Med., 1891, xlviii, 47.
(104) SIMPSON, M. E. AND J. M. DEMING. Folia Haemat., Teil I, 1927, xxxiv, 103.
(105) RICHTER, M. N. Arch. Int. Med., 1925, xxxvi, 13.
(106) DOMINICI, H. In V. CORNIL AND L. RANVIER. Histologie Pathologique, Alcan-Paris, ed. 3, 1902, ii, 614.
(107) LONGCOPE, W. T. Central. Bakt., I Abt., Ref., 1906, xxxvii, 23 and 112.
(108) HEDINGER, E. Frankfurter Zeitschr. f. Path., 1907, i, 527.
(109) OEHME, C. Münch. med. Wochenschr., 1909, lvi, 446.
(110) ASKANAZY, M. Virchow's Arch. path. Anat., 1915, ccxx, 257.
(111) FISCHER, O. VON. Frankfurter Zeitschr. f. Path., 1917, xx, 347.
(112) ASCHENHEIM, E. AND E. BENJAMIN. Deutsch. Arch. f. klin. Med., 1909, xcvii, 529.
(113) WEIDENREICH, F. Ergebn. d. Anat., 1909, xix, 776, 842.

V. BONE MARROW AS AN ORGAN.

(114) STOCKARD, C. R. AND G. N. PAPANICOLAOU. Amer. Journ. Anat., 1917, xxii, 225.
(115) ISAACS, R. AND A. C. DANIELIAN. Amer. Journ. Med. Sci., 1927, clxxiv, 70.
(116) BUNTING, C. H. AND J. HUSTON. Journ. Exper. Med., 1921, xxxiii, 593.
(117) GOLDSCHEIDER, A. AND P. JACOB. Zeitschr. klin. Med., 1894, xxv, 373.
(118) RUBINSTEIN, H. Zeitschr. klin. Med., 1901, xlii, 161.
(119) JONES, C. P. Brit. Med. Journ., 1905, i, 409.
(120) WOLOWNIK, B. Zeitschr. klin. Med., 1905, lvi, 529.

(121) BÄTGE, R. Histologische Untersuchungen über das spongiöse Knochenmark in verschiedenem Lebensalter, Fiencke, Kiel, 1908, (Inaug.-Diss. Univ. z. Erlangung.).
(122) LOSSEN, J. Virchow's Arch. path. Anat., 1910, cc, 258.
(123) SUZUKI, M. Scientific reports from the government institute for infectious diseases. Tokyo Imperial Univ., 1925, iv, 259.
(124) ARNETH. Deutsch. med. Wochenschr., 1925, li, 1350.
(125) SCHILLING, V. Deutsch. med. Wochenschr., 1925, li, 261, 344, 467, 516, 598.
(126) BANTZ, R. Vergleichende Untersuchungen über Blutbild und Knochenmark im Leben und nach dem Tode, mit besonderer Berücksichtigung der Kernverschiebung. Inaug.-Diss., Berlin, 1922.
(127) YAMAMOTO, T. Virchow's Arch. path. Anat., 1925, cclviii, 62.
(128) DOAN, C. A. AND L. G. ZERFAS. Journ. Exper. Med., 1927, xlvi, 511.
(129) SABIN, F. R. AND C. A. DOAN. Proc. Soc. Exper. Biol. and Med., 1927, xxv, 121.
(130) WETZEL, G. Anat. Anz., 1920–21, liii, Ergängzungsheft, p. 82.
(131) SCHILLING, V. Das Blutbild und seine klinische Verwertung. 5th/6th ed., Jena, Gustav Fischer, 1926.
(132) DOAN, C. A., L. G. ZERFAS, S. WARREN AND O. AMES. Journ. Exper. Med., 1928, xlvii, 403.
(133) HORWITZ, K. Wien. med. Wochenschr., 1904, liv, 1450, 1499, 1543, 1582, 1631.
(134) LATEINER-MAYERHOFER, M. Zeitschr. f. Kinderheilk., 1913–14, x, 152.
(135) WRIGHT, J. H. Journ. Morphol., 1910, xxi, 263.
(136) FRANK, E. Ergebn. d. ges. Med., 1922, iii, Pt. 2, 171.
(137) ANSELMINO, K. I. Virchow's Arch. path. Anat., 1926, cclxxi, 766.
(138) EVANS, H. E. Amer. Journ. Physiol., 1915, xxxvii, 243.
(139) WISLOCKI, G. B. Bull. Johns Hopkins Hosp., 1921, xxxii, 132.
(140) DOAN, C. A. Journ. Exper. Med., 1926, xliii, 289.
(141) PEABODY, F. W. AND G. O. BROUN. Amer. Journ. Path., 1925, i, 169.
(142) BALLIN, M. AND P. F. MORSE. Journ. Amer. Med. Assoc., 1927, lxxxix, 1671.
(143) WEILL, P. Virchow's Arch. path. Anat., 1919, ccxxvi, 212.
(144) FRANCO, E. E. AND A. FERRATA. Arch. per le sci. med., 1919, xlii, 109.
(145) FERRATA, A. Le Emopatie, Vol. I, Soc. Editrice Libraria, Milano, 1918.
(146) RICHTER, M. N. Amer. Journ. Med. Sci., 1925, clxix, 336.
(147) JORDAN, H. E. Virginia Med. Monthly, 1927, liii, 776.
(148) DOAN, C. A. AND F. R. SABIN. Journ. Exper. Med., 1926, xliii, 839.
(149) CARREL, A. AND A. H. EBELING. Journ. Exper. Med., 1926, xliv, 261.
(150) CARREL, A. AND A. H. EBELING. Journ. Exper. Med., 1926, xliv, 285.
(151) LEWIS, M. R. AND W. H. LEWIS. Carnegie Inst. of Washington, Pub. no. 363, Contrib. Embryol., 1926, xviii, 95.
(152) GARDNER, L. U. AND D. T. SMITH. Amer. Journ. Path., 1927, iii, 445.
(153) LEWIS, W. H. Arch. f. exper. Zellforsch., 1927, iv, 442.

(154) WEIDENREICH, F. Münch. med. Wochenschr., 1912, lix, 2601.
(155) WEILL, P. Arch. f. mikroskop. Anat., 1919–1920, xciii, 1.
(156) WEILL, P. Arch. f. mikroskop. Anat., 1919–1920, xciii, 82.
(157) BLOOM, W. Folia Haematol., Teil I, Archiv., 1926, xxxiii, 122.
(158) SABIN, F. R., C. A. DOAN AND R. S. CUNNINGHAM. Carnegie Inst. of Washington, Pub. no. 361, Contrib. Embryol., 1925, xvi, 125.
(159) SABIN, F. R. AND C. A. DOAN. Journ. Exper. Med., 1927, xlvi, 627.
(160) ONO, M. Scientific reports from the Government Institute for Infectious Diseases, Tokyo, 1926, v, 471.
(161) BRANNAN, D. Bull. Johns Hopkins Hosp., 1927, xli, 104.
(162) SALEEBY, E. R. Amer. Journ. Path., 1925, i, 69.
(163) LANG, F. J. Z. f. Mikr.-anatom. Forschung, 1926, iv, 417.
(164) SACERDOTTI, C. AND G. FRATTIN. Virchow's Arch. path. Anat., 1902, clxviii, 431.
(165) MAXIMOW, A. Beitr. z. path. Anat., 1907, xli, 122.
(166) RIBADEAU-DUMAS, L. AND G. ROUSSY. Compt. Rend. Soc. Biol., 1908, lxv, 333.
(167) GLASER, F. Med. Klinik, 1922, xviii, 331.
(168) GLASER, F. Klin. Wochenschr., 1923, ii, 1598.
(169) GLASER, F. Münch. med. Wochenschr., 1924, lxxi, 674.
(170) GLASER, F. AND P. BUSCHMANN. Deutsch. med. Wochenschr., 1923, xlix, 243.
(171) MÜLLER, E. F. Münch. med. Wochenschr., 1922, lxix, 1506.
(172) GLASER, F. AND P. BUSCHMANN. Med. Klin., 1923, xix, 1144.
(173) MUIR, R. Brit. Med. Journ., 1898, ii, 604.
(174) BUCHNER, H. Berl. klin. Wochenschr., 1890, xxvii, 673.
(175) ROEMER, F. Berl. klin. Wochenschr., 1891, xxviii, 886.
(176) ROEMER, F. Berl. klin. Wochenschr., 1891, xxviii, 1189.
(177) HORBACZEWSKI, J. Sitzungsb. d. k. k. Wiener Akad. der Wissensch., 1891, Bd. c, Abt. III, 101, Ref. B. (Abstr. Deutsch. med. Wochenschr., 1892, lxxx, 772.)
(178) LÖWIT, M. Studien zur Physiologie und Pathologie des Blutes und der Lymphe. Jena, Gustav Fischer, 1892.
(179) BACON, D. K., F. O. NOVY AND H. H. EPPLER. Arch. Int. Med., 1922, xxx, 229.
(180) COHN, E. J., G. R. MINOT, J. F. FULTON, H. F. ULRICHS, F. C. SARGENT, J. H. WEARE AND W. P. MURPHY. Journ. Biol. Chem., 1927, lxxiv, lxix.
(181) ZADEK, I. Zeitschr. f. klin. Med., 1926, ciii, 646.
(182) ROUS, P. AND O. H. ROBERTSON. Journ. Exper. Med., 1917, xxv, 651.
(183) ROBERTSON, O. H. AND P. ROUS. Journ. Exper. Med., 1917, xxv, 665.
(184) WELO, L. A. AND O. BAUDISCH. Journ. Biol. Chem., 1925, lxv, 215.
(185) WELO, L. A. AND O. BAUDISCH. Science, 1925, lxii, 311.
(186) MOLDAWSKY, I. Klin. Wochenschr., 1927, vi, 1998.
(187) DREW, A. H. AND J. C. MOTTRAM. Lancet, 1921, ii, 1202.
(188) KOESSLER, K. K. AND S. MAURER. Journ. Amer. Med. Assoc., 1927, lxxxix, 768.

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A METHOD FOR MAKING PERMANENT PREPARATIONS OF SUPRAVITALLY STAINED BLOOD CELLS.

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Two principal difficulties are encountered in the study of supravitally stained blood cells. In the first place, the preparations can only be observed over a relatively short space of time and it is difficult to make camera-lucida drawings and accurate observations, because the cells are not anchored in position, but move about of their own activity, or passively by reason of fluid currents in the plasma or suspending medium. Secondly, there is always more or less pathological change even when the observations are made in a carefully regulated warm chamber. One is, indeed, studying a protracted death on the part of the cells, and it is always difficult to decide at just what point the observations should be discontinued, in order that the accusation of describing changes which are not paralleled under living and normal conditions may be avoided.

The most useful supravital dye is Janus green. It was introduced by Michaelis in 1899, but subsequently there were several Janus greens on the market which were not effective, so that this method of revealing mitochondria did not come into general use until Bensley ('11) found that it was essential to employ a Janus green having exactly the formula prescribed by Michaelis. Other workers had been careless in this respect. The right Janus green is diethylsafranin-azodimethylanilin chloride, which may be identified by certain simple chemical tests (Cowdry, '18). This dye was successfully applied to human blood cells by Cowdry ('14), and has since been widely used in hematological studies. The other dye most commonly employed for blood cells is neutral red. We owe its use in this connection primarily to Renaut ('07). Another useful dye, although less frequently applied in this connection, is brilliant cresyl blue, first recommended for the investigation of blood cells by Levaditi ('01).

The following method permits the making of permanent prepara-

tions, at any desired stage of the process of supravital staining, which may be studied at once or may be set aside for reference at any time.

1. Preparation of the slides. One per cent solutions of Janus green and neutral red are made, each in absolute alcohol. After an hour, these solutions are filtered to remove any undissolved particles of dye. The usual precautions are observed in cleaning a number of slides. Three drops, from a pipette with a bore of approximately 1 mm., of either Janus green or neutral red solution are placed near the end of a slide. These are smeared the length of the slide with the end of another slide in the same way that one would make a blood smear. The amount of stain deposited may be regulated by the angle at which the second slide is held and the speed of movement. The alcohol evaporates almost immediately and a fine and even film of dye is thus secured. This technique of preparing the slides was first devised by Levaditi ('01) for the supravital coloration of blood cells with brilliant cresyl blue. He also made durable preparations of blood stained with this dye by drying in air and covering with balsam.

The slides may likewise be prepared for double staining with both Janus green and neutral red. In this case equal quantities of the dye solutions are drawn up into a single pipette and are there mixed together. Or the proportions of the stain may be varied at will, in accordance with the relative intensities of coloration wanted in the final preparation. Smears of the mixture are made in the same way as the single dyes.

2. Addition of the blood. The warmed surface of the prepared slide is touched to blood of the required amount as it appears at a small cut in the skin. The blood obtained in this way is immediately covered with a clean and warmed cover-glass on which slight pressure is exerted, causing it to spread properly and retarding coagulation. The edges of the cover-glass are sealed with melted vaselin, and the preparation is observed in a warm chamber (37.5°C.) with the aid of good light and a 1.5 Zeiss apochromatic objective and no. 6 compensating ocular.

3. Appearance of fresh preparations. In such a preparation of human blood stained with Janus green, some of the mitochondria are colored by the time the slide can be examined. The mitochondria of the small lymphocytes are the first to stain. In other leucocytes

the stain does not reach its greatest intensity until after ten or fifteen minutes. The supravital staining of human blood cells with this dye has been described in detail by Cowdry ('14), who allowed a small amount of blood to mix with a solution of the dye in 0.85 per cent aqueous sodium chloride. Since this method gives the same results as his with rather less pathological change, the actual process of staining does not require further description here.

When both the Janus green and neutral red are applied to human blood, the mitochondria and neutral-red granules in the leucocytes are colored in the same cells. This gives a true double supravital stain. The mitochondria in the small lymphocytes take up the dye first, as in the above-mentioned preparations stained only with Janus green. Very few neutral-red granules appear in the lymphocytes, and these are confined to peripheral cytoplasm. This relation of abundant mitochondria and a few granules stainable with neutral red is reversed in the granulocytes in which the mitochondria are of rare occurrence and are seen only with difficulty, owing to the fact that they are obscured by the specific granulations. Both the mitochondria and neutral-red granules are numerous in the large mononuclear leucocytes. It is possible to distinguish the neutral-red-stainable granules and the mitochondria side by side in individual platelets.

In cells other than the small lymphocytes the two dyes when used together generally act simultaneously. It sometimes happens, however, that the Janus-green coloration will appear first and will be followed shortly by the neutral red, but this order of appearance may be reversed.

In avian blood both the mitochondria and the neutral-red granules may be seen in the erythrocytes. The leucocytes and blood platelets present the same general appearance as in mammalian blood when colored by the double stain. In blood of canaries parasitized with Plasmodium praecox, the mitochondria and the granules stainable with neutral red can be seen in the schizonts and gametocytes. The special strain of Plasmodium praecox examined was obtained through the kindness of Dr. R. W. Hegner.

After expressing a small drop of fresh bone marrow from the rib of a guinea-pig and placing it upon a slide, the same methods may be used, although more pressure on the cover-glass is required than with the blood preparations in order that an even film may be secured. Or-

dinarily, fifteen to twenty minutes suffice to insure good staining of both mitochondria and neutral-red granules in bone-marrow cells.

4. Final preparation of the slide. When the mitochondria or neutral-red granules have become sufficiently stained, the vaselin is wiped from the edges of the cover-glass, which is then removed by slipping it toward the nearest edge of the slide. If it is detached in this way, the blood is spread evenly upon both the slide and cover-glass, and two preparations from the same drop of blood result.

The slide and cover-glass are then quickly dried in air. If this is done slowly, much distortion takes place. The drying may be completed in a vacuum desiccator for from two to four hours or by shaking in several changes of anhydrous ether. This treatment with ether is particularly necessary with bone marrow, for it removes the fat as well as the water. The slide is then placed in xylol to clear the cells and to remove any vaselin which may be still adherent. Only a drop of balsam and a cover-glass are needed to complete the preparation. Balsam seems preferable to cedar oil, because, owing to its slight acidity, it brings out the color of the neutral-red granules more sharply.

5. Permanency of the preparations. Preparations of guinea-pig bone marrow were exposed to the light of a carbon arc lamp for four hours and showed no fading. These slides were subjected to both the light and an accompanying temperature of about 45°C. Preparations of human and avian blood exposed to the same heat and light for five consecutive hours showed some fading of the Janus green, but not of the neutral red. Under ordinary conditions, such supravital preparations retain their colors undiminished for several months.

BIBLIOGRAPHY.

Bensley, R. R. 1911 Studies on the pancreas of the guinea-pig. Am. Jour. Anat., vol. 12, pp. 297–388.

Cowdry, E. V. 1914 The vital staining of mitochondria with Janus green and diethylsafranin in human blood cells. Inter. Monat. f. Anat. u. Physiol., Bd. 31, S. 267–286.

—— 1918 The mitochondrial constituents of protoplasm. Contrib. to Embryol., Carnegie Inst. Wash., no. 25, pp. 39–160.

Levaditi, C. 1901 Un cas de leucémie myélogène. Jour. de Physiol. et de Path. Gen., T. 3, pp. 424–438.

Michaelis, L. 1899 Die vitale Färbung, eine Darstellungsmethode der Zellgranula. Arch. f. mikr. Anat., Bd. 55, S. 558–575.

Renaut, J. 1907 Les cellules connectives rhagiocrines. Arch. d'anat. micr., T. 9, pp. 495–606.

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CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

III. PERIODIC AND PROGRESSIVE VARIATIONS IN NORMAL RABBITS.

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The results of a large series of determinations of calcium and inorganic phosphorus in the blood of normal rabbits were analyzed in previous papers (1, 2) from the standpoint of grouped material with a view to establishng certain basic facts concerning the amounts of these substances in the blood, the relations that obtain, and the extent of the variations that occur under given experimental conditions. The results have been analyzed also with respect to the values obtained for consecutive examinations of a given group of animals with especial reference to the occurrence of periodic and progressive variations in the calcium and inorganic phosphorus content of the blood. This aspect of the investigation will be presented in the present paper.

Material and Methods.

The data used in this paper are derived from experiments which were described in detail in the two preceding papers (1, 2). For present purposes, it is sufficient to say that we have used data from 5 groups of normal, male rabbits differing somewhat as to age, breed, the time covered by the observations, and the frequency with which blood examinations were made. The pertinent features of the experiments may be summarized as follows:

Group	No. of animals	No. of examinations	Observation period
I	10	216	Oct. 8, 1926, to May 18, 1927
II	5	85	Dec. 1, 1926, to June 8, 1927
III	10	143	Jan. 14, to July 1, 1927
IV	11	153	Mar. 11, to July 1, 1927
V	111	111	Oct. 8, 1926, to June 8, 1927

Between October 8, 1926, and July 1, 1927, determinations of calcium and inorganic phosphorus were made on 708 samples of blood from 147 rabbits. Group V was composed of 8 subgroups (2), the animals of which were examined only once; the other animals were examined repeatedly.

RESULTS.

The results are presented in the form of tabulated summaries and a series of text-figures. Tables I to VI give the actual values obtained for successive determinations of calcium and inorganic phosphorus as well as the relations between the two substances. The curves in Text-figs. 1 to 9 represent values that have been smoothed by the formula $\frac{A + 2B + C}{4}$.

TABLE I.

Group I. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 8	13.74 ± .198	0.93	6.79	5.18 ± .189	0.89	17.18
" 15	13.22 ± .215	1.01	7.65	4.55 ± .098	0.46	10.11
" 21	15.39 ± .247	1.16	7.53	4.23 ± .168	0.79	18.68
" 29	15.15 ± .215	1.01	6.64	4.15 ± .087	0.41	9.88
Nov. 5	16.07 ± .363	1.70	10.56	4.77 ± .141	0.66	13.84
" 12	13.94 ± .26	1.22	8.78	5.02 ± .115	0.54	10.76
" 19	15.16 ± .133	0.62	4.08	4.98 ± .109	0.51	10.24
" 26	14.64 ± .211	0.99	6.78	4.70 ± .115	0.54	11.49
Dec. 3	15.19 ± .294	1.38	9.08	4.52 ± .094	0.44	9.73
" 10	15.48 ± .136	0.64	4.13	4.26 ± .094	0.44	10.33
" 17	15.48 ± .198	0.93	6.00	4.10 ± .147	0.69	16.83
" 31	15.90 ± .094	0.44	2.77	4.56 ± .070	0.33	7.24
Jan. 14	15.4 ± .100	0.47	3.05	4.35 ± .113	0.53	12.18
" 28	16.9 ± .209	0.98	5.81	3.94 ± .060	0.28	7.11
Feb. 11	15.3 ± .149	0.70	4.58	4.14 ± .062	0.29	7.00
" 25	15.6 ± .122	0.57	3.65	4.15 ± .092	0.43	10.36
Mar. 11	15.8 ± .108	0.51	3.20	3.89 ± .049	0.23	5.91
" 25	15.6 ± .109	0.51	3.27	3.84 ± .045	0.21	5.47
Apr. 8	15.3 ± .173	0.81	5.29	3.75 ± .100	0.47	12.53
" 22	15.7 ± .207	0.92	5.86	3.43 ± .065	0.29	8.45
May 4	15.5 ± .119	0.53	3.42	3.25 ± .063	0.28	8.62
" 18	17.6 ± .119	0.53	3.01	3.95 ± .047	0.21	5.32

TABLE II.

Group II. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Dec. 1	13.9 ± .368	0.82	8.79	3.83 ± .084	0.28	7.23
Jan. 5	15.3 ± .244	0.81	5.29	5.03 ± .133	0.44	8.75
" 19	15.6 ± .184	0.61	3.92	4.66 ± .056	0.19	3.99
Feb. 4	17.2 ± .235	0.78	4.53	4.38 ± .118	0.39	8.90
" 11*	16.6 ± .965	3.20	19.28	4.41 ± .103	0.34	7.71
" 16	15.4 ± .201	0.67	4.32	4.32 ± .133	0.44	10.19
" 28	15.6 ± .253	0.84	5.38	3.85 ± .045	0.15	3.89
Mar. 2	15.0 ± .214	0.71	4.73	4.07 ± .021	0.07	1.74
" 9	14.7 ± .362	1.20	8.16	4.10 ± .130	0.43	10.49
" 16	14.8 ± .193	0.64	4.32	3.64 ± .078	0.26	7.14
" 23	14.8 ± .089	0.30	2.00	3.72 ± .066	0.22	5.91
Apr. 6	15.5 ± .187	0.62	4.06	4.53 ± .181	0.60	13.25
May 4	15.4 ± .259	0.86	5.58	3.87 ± .118	0.39	10.08
" 6	15.2 ± .193	0.64	4.21	4.21 ± .072	0.24	5.70
" 11	17.0 ± .274	0.91	5.35	3.90 ± .075	0.25	6.41
" 25	16.6 ± .145	0.48	2.89	3.76 ± .094	0.31	8.24
June 8	15.8 ± .259	0.86	5.44	3.61 ± .115	0.38	10.53

* This group contained one value 22.8 and another 13.8.

TABLE III.

Group III. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 14	14.8 ± .181	0.89	6.01	5.75 ± .104	0.51	8.87
" 26	16.3 ± .256	1.26	7.73	5.24 ± .112	0.55	10.50
Feb. 9	15.0 ± .122	0.57	3.80	5.33 ± .113	0.53	9.94
" 18	15.3 ± .151	0.71	4.64	5.13 ± .087	0.41	7.99
Mar. 4	15.2 ± .159	0.78	5.13	4.74 ± .083	0.41	8.65
" 18	15.3 ± .119	0.56	3.66	4.85 ± .088	0.39	8.04
" 30	14.8 ± .119	0.56	3.78	5.11 ± .094	0.44	8.61
Apr. 13	14.8 ± .109	0.51	3.45	4.44 ± .092	0.43	9.68
" 27	14.9 ± .109	0.51	3.42	4.34 ± .079	0.37	8.53
May 11	16.4 ± .143	0.67	4.09	4.17 ± .115	0.54	12.95
" 25	16.3 ± .128	0.60	3.68	3.93 ± .111	0.52	13.23
June 8	16.6 ± .119	0.56	3.37	3.70 ± .094	0.44	11.89
" 17	17.1 ± .267	1.25	7.31	4.29 ± .092	0.43	10.02
July 1	17.4 ± .122	0.57	3.28	4.28 ± .107	0.50	11.68
Sept. 15	14.9 ± .186	0.87	5.84	4.24 ± .100	0.47	11.08
Oct. 19	15.9 ± .256	1.20	7.55	3.99 ± .109	0.51	12.78

TABLE IV.

Group IV. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Mar. 11	15.2 ± .195	0.96	6.32	5.90 ± .126	0.62	10.51
" 18	15.1 ± .110	0.54	3.58	5.55 ± .112	0.55	9.91
" 25	14.9 ± .087	0.43	2.89	6.23 ± .094	0.46	7.38
Apr. 1	15.2 ± .115	0.57	3.72	5.84 ± .126	0.62	10.62
" 8	15.5 ± .110	0.54	3.48	5.01 ± .100	0.49	9.78
" 15	15.3 ± .122	0.60	3.92	4.48 ± .087	0.43	9.60
" 22	15.0 ± .117	0.58	3.83	4.93 ± .107	0.53	10.67
" 29	15.2 ± .230	1.13	7.43	4.71 ± .087	0.43	9.13
May 6	15.6 ± .159	0.78	5.00	4.61 ± .107	0.53	11.39
" 13	16.2 ± .106	0.52	3.21	4.51 ± .122	0.60	13.30
" 25	17.0 ± .146	0.72	4.24	4.58 ± .075	0.35	7.64
June 8	16.8 ± .120	0.59	3.51	4.10 ± .075	0.37	9.02
" 17	17.6 ± .191	0.94	5.34	4.57 ± .069	0.34	7.66
July 1	17.2 ± .068	0.34	1.95	4.42 ± .106	0.52	11.77
Sept. 15	15.4 ± .195	0.96	6.23	4.39 ± .073	0.36	8.20
Oct. 25	15.2 ± .147	0.69	4.54	4.28 ± .075	0.35	10.51

TABLE V.

Group. V. Values for Consecutive Monthly Groups.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 8	13.7 ± .198	0.93	6.79	5.18 ± .189	0.89	17.18
Nov. 17	14.7 ± .066	0.31	2.11	5.22 ± .124	0.58	11.11
Jan. 7–14	15.3 ± .169	1.15	7.52	5.06 ± .135	0.92	18.18
Feb. 4	17.5 ± .097	0.48	2.74	5.33 ± .146	0.72	13.51
Mar. 11	15.0 ± .128	1.08	7.20	5.48 ± .108	0.91	16.61
Apr. 29	15.1 ± .155	0.73	4.83	5.53 ± .068	0.32	5.77
May 25	16.2 ± .200	0.89	5.49	5.81 ± .245	1.09	18.76
June 8	17.5 ± .230	0.97	5.53	5.92 ± .136	0.57	9.63

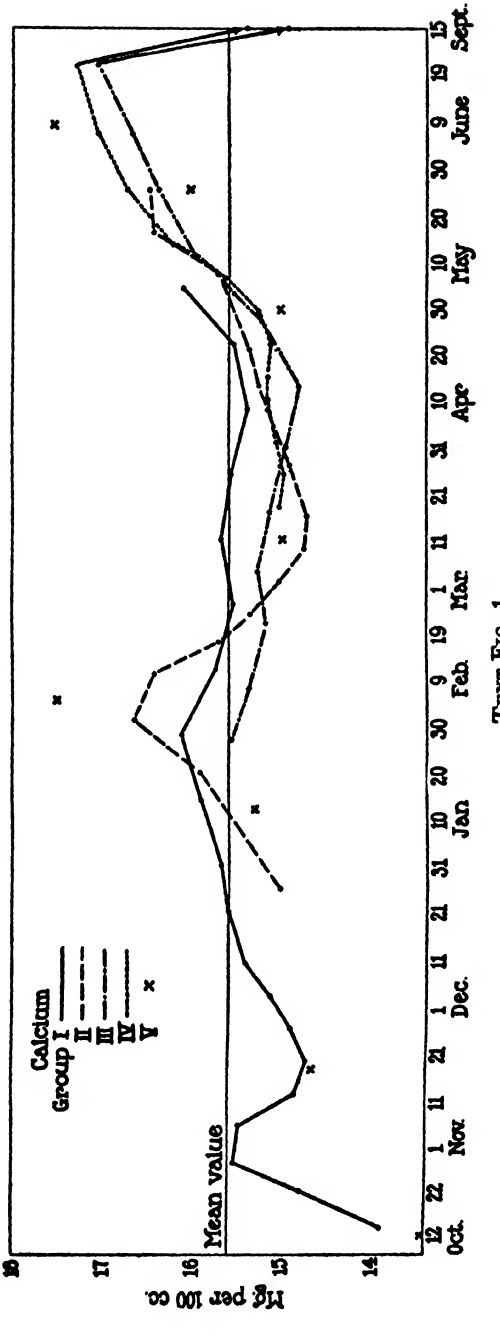
TABLE VI.

Group Means for Consecutive Determinations: Sum, Product, Calcium-Inorganic Phosphorus Ratio, Product-Sum Ratio, and Sum of Both Ratios.

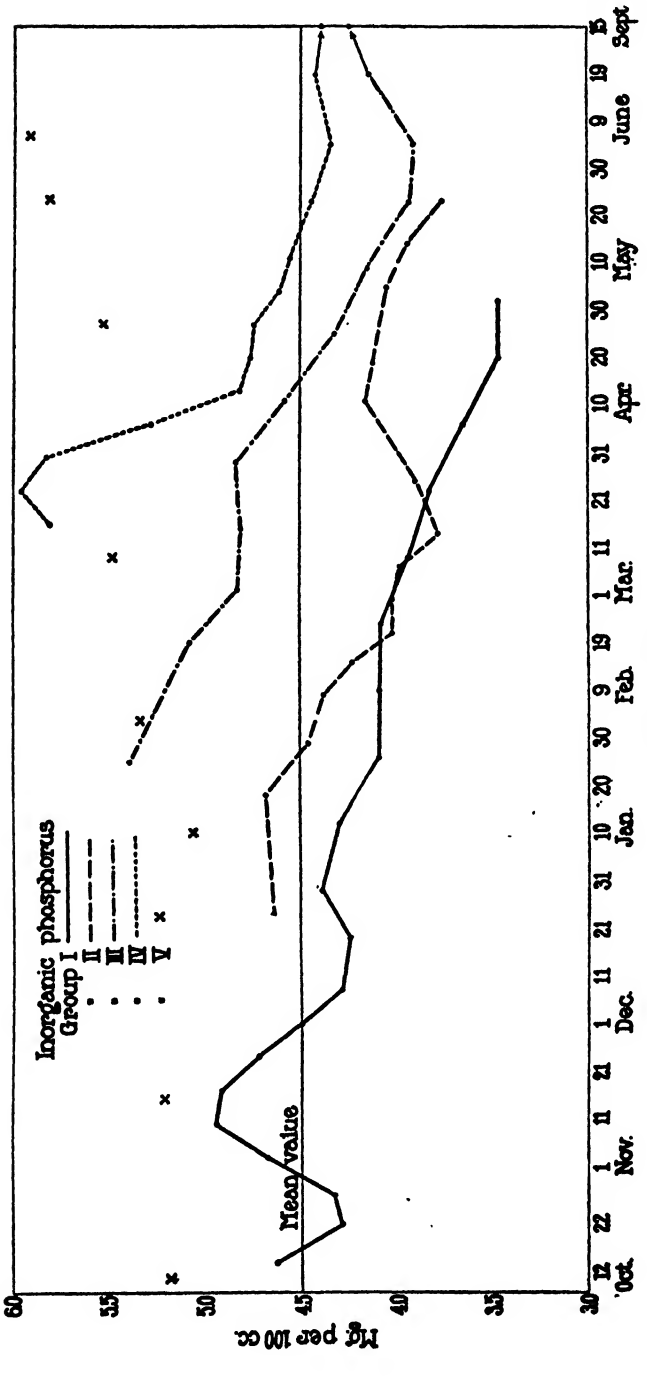
Group	Date	Sum	Product	Calcium-phosphorus ratio	Product-sum ratio	Sum of two ratios
I	Oct. 8	18.9	71.2	2.65	3.76	6.41
	" 15	17.8	60.2	2.91	3.39	6.30
	" 21	19.6	65.1	3.64	3.32	6.96
	" 29	19.3	62.9	3.65	3.26	6.91
	Nov. 5	21.2	78.3	3.44	3.70	7.14
	" 12	19.0	70.0	2.77	3.69	6.46
	" 19	20.1	75.5	3.04	3.75	6.79
	" 26	19.3	68.8	3.12	3.56	6.68
	Dec. 3	19.7	68.7	3.36	3.49	6.85
	" 10	19.7	65.9	3.63	3.34	6.97
	" 17	19.6	63.5	3.77	3.24	7.01
	" 31	20.5	72.5	3.48	3.54	7.02
	Jan. 14	19.8	67.1	3.54	3.39	6.93
	" 28	20.9	66.6	4.29	3.19	7.48
	Feb. 11	19.4	63.2	3.68	3.26	6.94
	" 25	19.7	64.7	3.75	3.28	7.03
	Mar. 11	19.7	61.3	4.01	3.12	7.13
	" 25	19.5	60.1	4.09	3.08	7.17
	Apr. 8	19.0	57.2	4.07	3.01	7.08
	" 22	19.1	53.9	4.58	2.82	7.40
	May 5	18.8	50.4	4.77	2.68	7.45
	" 18	21.8	70.3	4.51	3.22	7.73
II	Dec. 1	17.8	53.4	3.64	3.00	6.64
	Jan. 5	20.3	77.0	3.04	3.78	6.82
	" 19	20.2	72.5	3.34	3.58	6.92
	Feb. 4	21.6	75.5	3.93	3.49	7.42
	" 11	21.0	72.3	3.72	3.44	7.16
	" 16	19.7	66.4	3.55	3.37	6.92
	" 28	19.4	59.9	4.04	3.09	7.13
	Mar. 2	19.0	60.9	3.67	3.19	6.86
	" 9	18.8	60.2	3.58	3.20	6.78
	" 16	18.4	53.7	4.06	2.92	6.98
	" 23	18.5	54.9	3.96	2.97	6.93
	Apr. 6	20.0	70.0	3.41	3.50	6.91
	May 4	19.3	59.7	3.98	3.09	7.07
	" 6	19.4	64.1	3.62	3.29	6.91
	" 11	20.9	66.2	4.35	3.17	7.52
	" 25	20.4	62.4	4.41	3.06	7.47
	June 8	19.4	56.9	4.36	2.93	7.29

TABLE VI—*Concluded.*

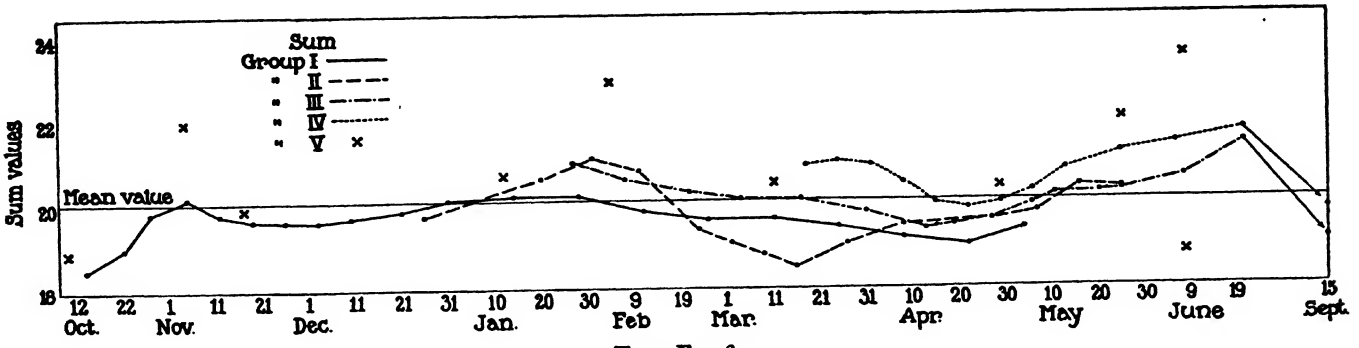
Group	Date	Sum	Product	Calcium-phosphorus ratio	Product-sum ratio	Sum of two ratios
III	Jan. 14	20.6	85.1	2.57	4.13	6.70
	" 26	21.5	85.4	3.11	3.97	7.08
	Feb. 9	20.3	80.0	2.81	3.94	6.75
	" 18	20.4	78.5	2.98	3.85	6.83
	Mar. 4	19.9	72.0	3.21	3.62	6.83
	" 18	20.2	74.2	3.15	3.67	6.82
	" 30	19.9	75.6	2.90	3.80	6.70
	Apr. 13	19.2	65.7	3.33	3.42	6.75
	" 27	19.2	64.7	3.43	3.37	6.80
	May 11	20.6	68.4	3.93	3.32	7.25
	" 25	20.2	64.1	4.15	3.17	7.32
	June 8	20.3	61.4	4.49	3.02	7.51
	" 17	21.4	73.4	3.99	3.43	7.42
	July 1	21.7	74.5	4.07	3.43	7.50
	Sept. 15	19.1	63.2	3.51	3.31	6.82
IV	Mar. 11	21.0	89.7	2.58	4.25	6.83
	" 18	20.7	83.5	2.71	4.05	6.76
	" 25	21.1	92.6	2.38	4.39	6.77
	Apr. 1	21.0	88.5	2.60	4.21	6.81
	" 8	20.5	77.7	3.09	3.79	6.88
	" 15	19.8	68.5	3.41	3.46	6.87
	" 22	19.9	74.0	3.05	3.71	6.76
	" 29	19.9	71.9	3.23	3.60	6.83
	May 6	20.2	71.8	3.38	3.55	6.93
	" 13	20.7	72.9	3.58	3.52	7.10
	" 25	21.6	77.9	3.71	3.61	7.32
	June 8	20.9	68.9	4.10	3.30	7.40
	" 17	22.2	80.4	3.85	3.62	7.47
	July 1	21.6	76.0	3.89	3.52	7.41
	Sept. 15	19.8	67.6	3.51	3.41	6.92
V	Oct. 8	18.9	71.5	2.72	3.74	6.46
	Nov. 17	19.9	76.4	2.84	3.84	6.68
	Jan. 12	20.3	76.9	3.14	3.76	6.90
	Feb. 4	22.9	93.5	3.35	4.07	7.42
	Mar. 11	20.5	82.0	2.82	3.98	6.80
	Apr. 29	20.4	83.0	2.84	4.06	6.89
	May 25	22.0	93.6	2.90	4.11	7.01
	June 8	23.5	104.0	2.98	4.41	7.39


Mg per 100 cc.
18
17
16
15
14
Mean value
Calcium
Group I
II
III
IV
V
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

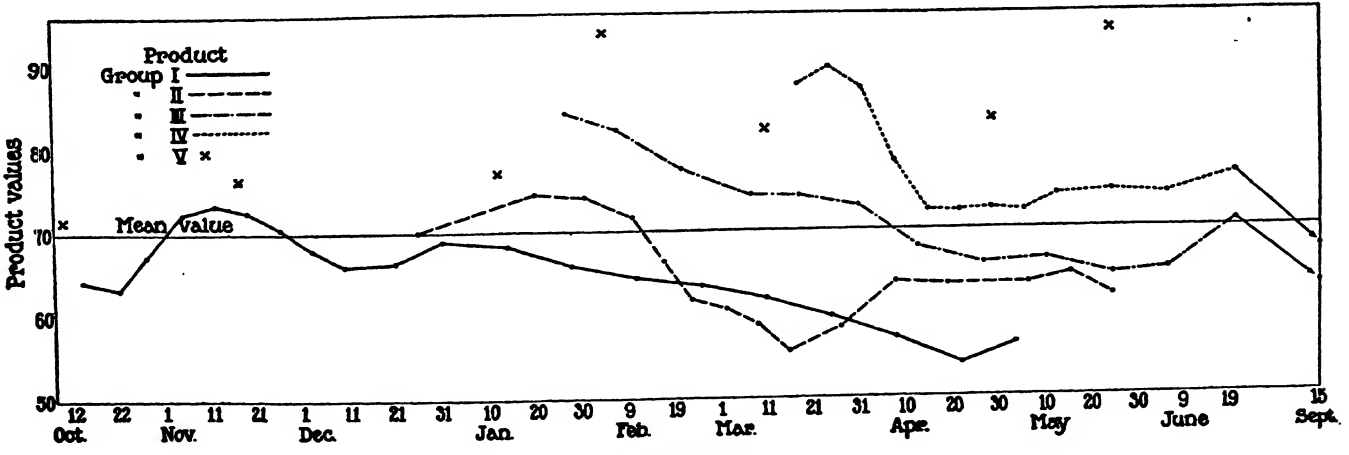
TEXT-FIG. 1.


Mg per 100 cc.
60
55
50
45
40
35
30
Inorganic phosphorus
Group I
II
III
IV
V
Mean value
12 Oct.
22
1 Nov.
11
21
1 Dec.
11
21
31
10 Jan.
20
30
9 Feb.
19
1 Mar.
11
21
31
10 Apr.
20
30
10 May
20
30
9 June
19
25 Sept.

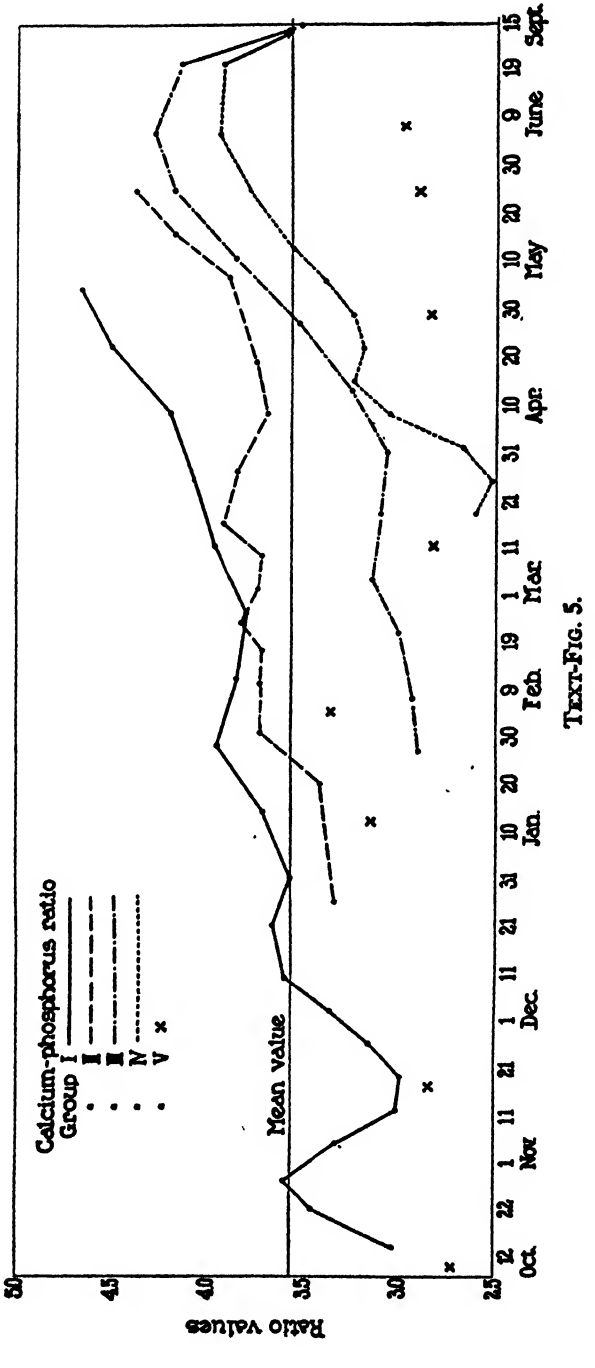
TEXT-FIG. 2.



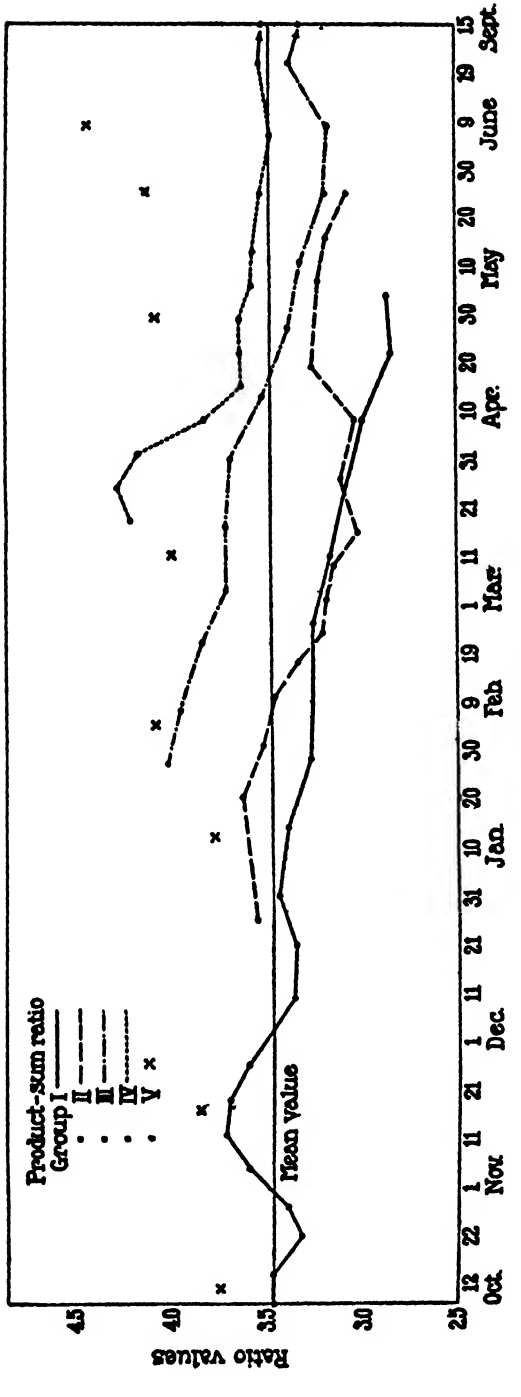
TEXT-FIG. 3.



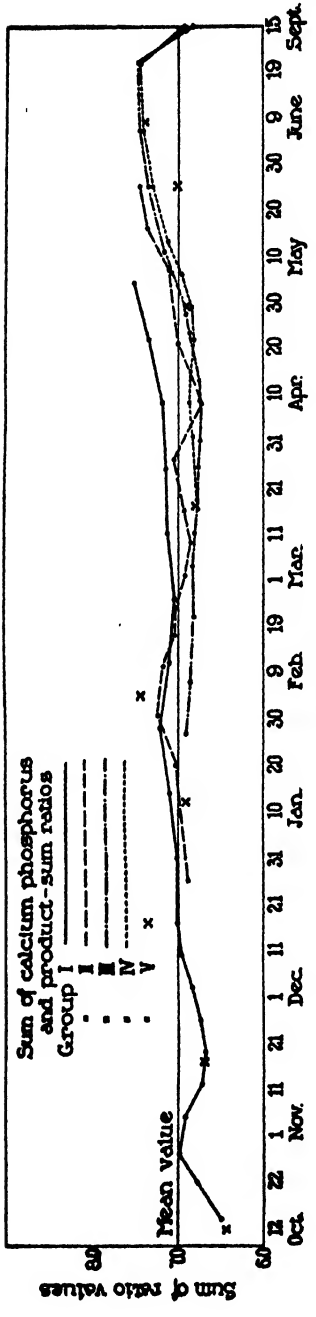
TEXT-FIG. 4.


Calcium-phosphorus ratio
Group I
II
III
IV
V
Mean value
Ratio values
50
45
40
35
30
25
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

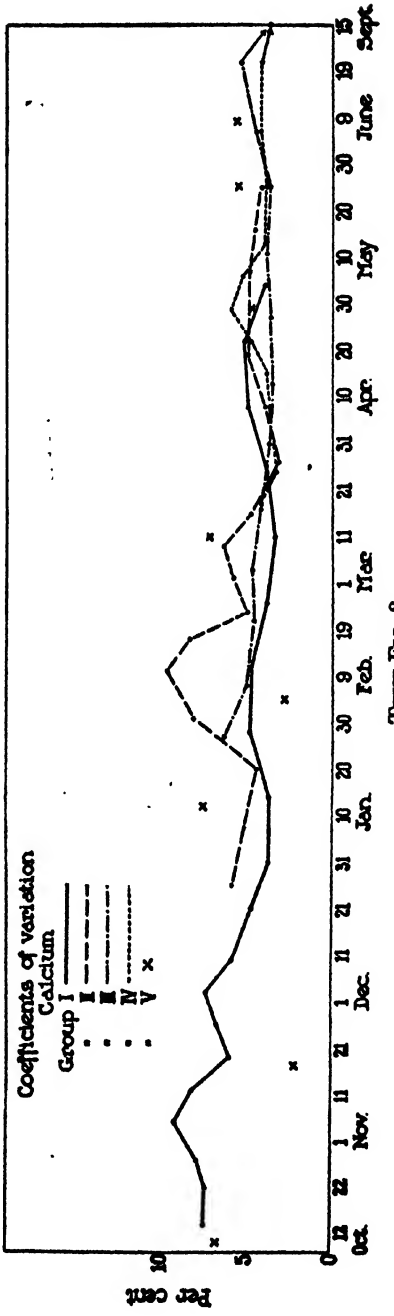
TEXT-FIG. 5.


Product-sum ratio
Group I
II
III
IV
V
Mean value
Ratio values
45
40
35
30
25
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 29 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

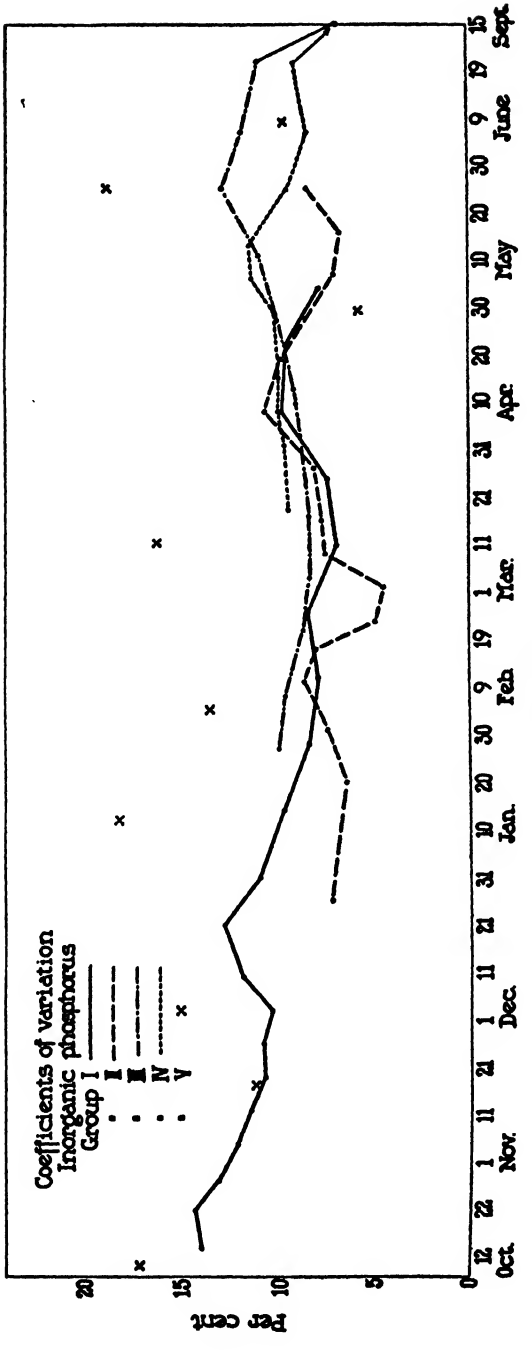
TEXT-FIG. 6.



TEXT-FIG. 7.



TEXT-FIG. 8.


Coefficients of variation
Inorganic phosphorus
Group I
II
III
IV
V
Per cent
0
5
10
15
20
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb Mar. Apr. May June Sept.

TEXT-FIG. 9.

DISCUSSION AND CONCLUSIONS.

An examination of the figures given in Tables I to VI and the curves in Text-figs. 1 to 9 shows at once that both the absolute values for calcium and inorganic phosphorus and the relative amounts of these substances exhibited an interesting series of variations in all of the animals studied. The changes observed in different groups of animals were not identical, but there were certain general features that were common to all, as may be seen by comparing the curves in Text-figs. 1 to 9. The course of events is more difficult to follow from the tabulated results.

In practically all instances, there are variations of two types. One of these is progressive in character and represents a gradual change of values from a lower to a higher, or from a higher to a lower level. This gradual change of level is accompanied in some instances by a clearly defined series of periodic variations, the positive and negative phases of which extend over periods of several weeks or months. In other cases, clearly defined periodic increases are absent, but the gradual change in level is marked by periods of stabilization at a given level, or by a temporary increase or decrease in the rate of change. The tabulated results show, however, that neither of these conditions represents a continuous movement in a given direction, but that the periodic variation is itself produced by a succession of pendulum-like fluctuations. Moreover, it will be seen that, to some extent, the progressive increase or decrease in values is in reality a long period variation as the high or low levels are not maintained indefinitely. This is particularly true of calcium as may be seen by reference to the tables and text-figures for Groups III and IV (Tables III and IV and Text-fig. 1).

From October until July, the general trend of calcium values is slightly upward (Tables I to V and Text-fig. 1). The outstanding feature of the condition shown by blood calcium is, however, the occurrence of a series of periodic increases and decreases. From October to December, 1926, consecutive observations were made on only one group of animals (Group I). These showed a gradual increase in calcium which reached a maximum about the last of October or the first of November (Table I and Text-fig. 1). This increase was fol-

lowed by a decrease which was succeeded in turn by a gradual and prolonged rise, terminating in a second maximum toward the end of January, 1927. A gradual fall in calcium then began and continued well into April. There was a suggestion of a secondary rise at the end of February and the first of March, but the third definite period of increase did not begin until the end of April or the first of May.

The variations in calcium shown by other groups of animals differed mainly with respect to the general level and the exact time of occurrence of maximum and minimum values. The conditions shown by Groups III and IV compare favorably with those of Group I (Tables III and IV and Text-fig. 1). The results for Groups III and IV are of especial interest in that they show that the period of increasing calcium which began in April or May continued through June, reaching a very high value on July 1. Unfortunately, no observations could be made on these animals during July and August, but by the middle of September, their calcium had again fallen to a point slightly below the mean normal level (15.6 mg.) or to values comparable with those for the preceding April and early May.

The interesting feature of the results for Group II is the extent of the variations shown (Table II and Text-fig. 1). The January-February maximum was considerably higher than that of Group I, while the March minimum was much lower. Moreover, the succeeding rise began while the calcium in the blood of other animals was still falling and well in advance of the rise in other groups.

Comparing the results for consecutive observations on animals caged in the laboratory (Groups I to IV) with those for animals examined as they were received from the dealer (Group V), it will be seen that, while the two sets of results agree with respect to the form of the variations, there is an interesting difference in the magnitude of the variations shown. In recently acquired animals, maximum values tended to be higher and minimum values lower, which suggests that the calcium content of the blood is subject to greater variation among animals living out of doors than among those caged in the laboratory.

The smoothed curves give the impression of a continuous or uninterrupted movement in a given direction which, with due allowance for technical inaccuracies and chance errors, is probably incorrect. A critical examination of the tabulated results will show that, in all prob-

ability, the prolonged rise and fall was accomplished by a series of short pendulum-like fluctuations with the general level rising or falling, as the case might be. This applies not only to calcium, but to all other values.

The situation presented by inorganic phosphorus is distinctly different from that of calcium (Tables I to V and Text-fig. 2). The striking feature of the results obtained for animals living under laboratory conditions is a marked and progressive decrease in inorganic phosphorus (Text-fig. 2); this is in sharp contrast with results for animals examined as they were received from the dealer. Available data for recently acquired animals show little or no change in inorganic phosphorus until January or February. There is a suggestion of a decrease in January and of a beginning rise in February. This rise was, however, comparatively slight and virtually ceased during March and April, to be resumed and continued through May and June.

Among the animals living under laboratory conditions, there are several features of the results that appear to be significant. Of these, the progressive decrease in inorganic phosphorus is most evident. It will be noted also that the results for each group of animals occupy a different level with comparatively little overlapping, and that the levels of successive groups rise in somewhat the same manner as the results for recently acquired animals examined at corresponding times (Text-fig. 2). Moreover, while all animals show a decrease in inorganic phosphorus, it will be seen that both the rate and extent of the decrease varied in different groups, and that in no case did the reduction proceed uninterrupted or at a uniform rate. In fact, each of the 4 groups of animals showed one or more periods during which the decrease in phosphorus ceased or gave place to an actual increase. This may be brought out by an analysis of the course of events in a given group of animals.

During November, the animals of Group I showed an abrupt increase in inorganic phosphorus extending over a period of 2 to 3 weeks with a return to the previous level about the first of December. This change coincided with a decrease and a subsequent rise in calcium. For 3 months thereafter, the decrease in phosphorus was comparatively slight, but during March and April it diminished at a more rapid rate, reaching the lowest level about the first of May. The last observation

on these animals gave a value that was higher than any that had been obtained since February which suggests that the phosphorus was again on the point of increase.

The distinctive feature of the results obtained for Group II is the marked decrease in inorganic phosphorus between January 5 and March 16, and the subsequent increase during March and April. In this instance, the change in inorganic phosphorus coincided with like changes in the calcium content of the blood.

The results for Groups III and IV show conditions that are again different. In the case of Group III, there was a reduction in inorganic phosphorus during the first 6 or 7 weeks; this was followed by an increase during March (Table III) and a second decrease which continued into June and was succeeded by a slight rise toward the end of June and the first of July. The striking feature of the change noted in Group IV is the marked reduction in inorganic phosphorus during the first 2 weeks of April. From this point on, the results compare favorably with those for Group III.

Considered as a whole, the results for inorganic phosphorus are somewhat confusing, due to the lack of agreement between recently acquired stocks and animals living under laboratory conditions, as well as to differences in the behavior of animals placed under observation at different times. Still, there is considerable evidence to show that inorganic phosphorus is subject to the action of influences which tend to raise or lower the level, as in the case of calcium, but these influences do not operate in precisely the same manner on animals confined in the laboratory and on those living out of doors. It is important to note that the change from outdoor to indoor life appears to be sufficient in most cases to initiate a decrease in the phosphorus content of the blood. In some instances, the immediate effect may be very slight, or even in the opposite direction, due perhaps to the fact that indoor conditions do not differ materially from those to which the animal has been accustomed (Group I); but, in other instances, the decrease in phosphorus may be marked (Group IV). Under such circumstances, the action of factors tending to increase the inorganic phosphorus of the blood may be completely obscured or accomplish no more than a reduction in the rate of decrease or a temporary stab-

ilization at a given level, such as occurred at several points in these experiments.

While it is reasonably certain that periodic variations in the trend of inorganic phosphorus occurred, definite movements can not be traced with the same degree of certainty as in the case of calcium. The indications are that there was a tendency toward lower values for animals of a given age, during the late fall and early winter months which was succeeded by a rise in January or February. The condition that prevailed during March and April is even more obscure, as some animals showed an accentuation of the decrease in inorganic phosphorus while others maintained a fairly high level. During May the trend was downward, and the final rise did not begin until June.

Applying the same principles of analyses to the values obtained for the sum and the product of calcium and inorganic phosphorus (Table VI and Text-figs. 3 and 4), it will be seen that, in general, these values reflect the variations shown by calcium and phosphorus respectively. This is particularly true of the sum, but the product shows a series of variations that is more clearly defined than those of phosphorus. In this case, the results obtained for recently acquired animals are of especial interest as they show a high value for February followed by much lower values for March and April and high values for May and June. In this respect, the results are in close agreement with those for calcium and for the sum of calcium and phosphorus.

The results for the ratio of calcium to inorganic phosphorus (Table VI and Text-fig. 5) disclose a condition that is almost the reverse of that shown by inorganic phosphorus. The general trend of values for all animals caged in the laboratory is upward, but variations in the absolute amounts of both calcium and phosphorus are reflected in these values.

Calcium-phosphorus ratio values for recently acquired animals show a gradual increase from October to February with a decided drop during March and April and a second slight rise during May and June. Animals in the laboratory show essentially the same conditions, if we disregard minor fluctuations. The chief difference between the two sets of values is that animals under continuous observation do not show a decided reduction in the ratio values for March and April, but a slight reduction or stabilization which is followed by a very

marked increase when the curve again turns upward. From observations on 2 groups of animals (Groups III and IV), it was found that the high values attained during this period of increase were eventually succeeded by a decrease. It is thus seen that, despite the progressive decrease in inorganic phosphorus, there is evidence of the occurrence of distinct periodic variations in the calcium-phosphorus equilibrium.

As a rule, the ratio of the product to the sum shows an inverse relation to the calcium-phosphorus ratio, and the sum or mean of the two values varies within very narrow limits or is virtually constant for a given set of conditions (1). An interesting exception to this rule is shown by the group of recently acquired animals (Table VI and Text-figs. 6 and 7). In this instance, it will be seen that the relation between values for the product-sum ratio and the calcium-phosphorus ratio is direct and not inverse. But, despite this difference, the quantitative relations are in close agreement with values obtained by repeated examination of given groups of animals, and these results give further evidence of the same series of periodic variations as was found by analysis of other values (Text-fig. 7).

The series of variations described above are, on the whole, so clearly shown by the curves that it would hardly seem necessary to resort to detailed statistical methods to determine whether variations of the order indicated represent actual differences in values or are within the range of probable variation due to random sampling. It is obvious that when a given change occurs gradually and by a series of pendulum-like swings, as in the present instance, nothing of importance can be learned by a comparison of successive values unless each value is based on a large series of observations. On the other hand, something may be gained by comparing values for given periods which represent a general trend or opposite phases of a supposed variation. A few comparisons of this kind will serve to indicate the probable significance of the results as a whole.

As the first example, we may compare the results obtained for calcium on Group I from November 12 to December 3 inclusive with those of December 17 to January 28 (Table I and Text-fig. 1). The mean values of all observations for these periods are 14.7 $\pm$ .128 and 15.9 $\pm$ .099 mg. respectively. As the difference of 1.2 $\pm$ .16 mg. is 7.5

times its probable error, it is certain that the difference between the values obtained for the two periods is significant.

There are even greater differences between results obtained for other groups during March and April, which in general represented a period of low calcium, and May, June, and July when the calcium rose to a much higher level. In the case of Group III, the mean value of all determinations from March 4 to April 27 inclusive is 15.0 ± .062 mg. and for May 11 to July 1, 16.7 ± .082 mg. which gives a difference of 1.7 ± .10. This difference is 17 times its probable error. The results for Group IV, comparing values for March 11 to April 1, and May 25 to July 1, are 15.1 ± .068 and 17.1 ± .073 mg. with a difference of 2.0 ± .10, which is 20 times its probable error. The March and April values for Group V give a mean of 15.0 ± .104 while the mean for May and June is 16.8 ± .180. In this case the difference of 1.8 ± .21 is 8.57 times the probable error.

Added significance is given to these results by the close agreement of the values obtained for different groups of animals at corresponding periods, as shown by the following tabulation of calcium values:

Group	Period of low values	Period of high values
III	15.0 ± .062	16.7 ± .082
IV	15.1 ± .068	17.1 ± .073
V	15.0 ± .104	16.8 ± .180

Less can be gained by a statistical analysis of the results for inorganic phosphorus. The progressive reduction in inorganic phosphorus among animals living under laboratory conditions is so evident that no further proof of the validity of this change is necessary. There are, however, a few instances in which a presumptive increase in inorganic phosphorus occurred in the course of the general downward movement, and the data bearing on these changes may be examined more critically. One of the best illustrations of a change of this kind is furnished by the increase in inorganic phosphorus which occurred in the animals of Group I during the middle of November. The observations for October 21 and 29 give a mean of 4.19 ± .099 mg. and those of November 12 and 19, 5.00 ± .078 mg. This difference is 6.43 times its probable error (.81 ± .126) and hence is definitely significant.

An analogous increase in inorganic phosphorus occurred in the animals of Group II between April 6 and May 11. The mean value for this period was 4.13 ± .075 mg. as compared with a mean of 3.88 ± .056 for March. As this difference (0.25 ± .09) is only 2.78 times its probable error, it may not be significant, but probably is.

A comparison of the values obtained for Group III on May 25 and June 8 with those for June 17 and July 1 gives a difference (0.48 ± .10) that is 4.8 times its probable error, and the October and November results for Group V compared with values for May and June give a difference (0.66 ± .18) that is 3.66 times the probable error. It is evident, therefore, that even in the case of inorganic phosphorus, there were instances in which a significant reversal of the dominant trend occurred.

Statistical comparisons of the results obtained for various measures of the relation between calcium and phosphorus have not been worked out in detail as it is evident that essentially the same situation obtains as in the case of calcium and phosphorus.

In this connection, however, some reference may be made to the degree of variation shown by the results obtained for individual animals at any given time as indicated by the coefficients of variation. With few exceptions, the coefficients of variation (Tables I to VI and Text-figs. 8 and 9) for consecutive determinations of both calcium and inorganic phosphorus are smaller than those for all observations on a given group of animals (1, 2) and they tend to diminish with successive observations. This, of course, indicates that the variation shown by the animals of a group at a particular time is less than that shown by the group during the course of the experiment and that group results tend to become more and more uniform.

There is, however, an evident relation between the variation of mean values and the magnitude of the coefficients. In general, it may be said that, so long as mean values remain comparatively constant, the coefficient of variation tends to be small, but the occurrence of either a decided increase or decrease in mean values is usually associated with an increase in the coefficient of variation. If the change in mean values is gradual and continuous, the coefficient tends to diminish until the magnitude of the mean values approaches the extreme limits of normal when the coefficient again increases. Further

more, while exceptionally high or low values may have a large coefficient of variation, it will be seen, by reference to Tables I to V, that if a high or low value is maintained, the coefficient is usually small. This is especially true of calcium.

From what has been said, it will be seen that there is no inherent relation between the magnitude of a mean value with respect to normal and its coefficient of variation. The relation found is one that indicates the occurrence of change, and in this sense the coefficient of variation becomes a valuable index of the stability of the animal organism with respect to particular conditions. Thus, a large coefficient or an increase in the coefficient of variation for a given series of calcium determinations indicates not only an uncertainty as to the true mean value, but is clearly indicative of an unstable condition and suggests the probable occurrence of a change, while a small coefficient or a decrease in the coefficient implies stability and uniformity of action.

The most important deduction to be drawn from the analysis of these results with respect to time, is that both the calcium and the inorganic phosphorus in the blood of normal rabbits are subject to progressive and periodic variations which effect not only the absolute but the relative amounts, or the equilibrium between the two substances which, within the limits of normal, is probably of more importance than absolute amounts. In like manner, periodic variations are more important in the present connection, than progressive variations as they provide a better basis for a study of any relation that may exist between changes in the chemical composition of the blood and susceptibility to disease.

The results reported agree with observations originally made by Hess and Lundagen (3) on calcium and inorganic phosphorus in the blood of infants and young children and with the results obtained by Grant and Gates (4) for calcium in the blood of normal rabbits, in so far as the occurrence of variations in one or the other of these substances is concerned. There are, however, certain differences. The variations found by Hess and Lundagen were confined largely to inorganic phosphorus, and no significant variations were found in adults. Moreover, there was a steady reduction in phosphorus from December to March

which was followed by a rise during May and June producing what was termed by them a seasonal tide.

In the experiments of Grant and Gates, the results for inorganic phosphorus were not analyzed. The highest values for calcium were obtained in May and November and the lowest in January, but no results were reported for February, June, July, or August.

In the experiments reported above, periodic variations were more clearly defined in the case of calcium than inorganic phosphorus, but the results differ from those of Grant and Gates as to the time of occurrence of maximum and minimum values. The results for inorganic phosphorus are more difficult to interpret, but it seems that for recently acquired stocks, the lowest value was obtained in January and that the increase began in February. There was, however, little or no further increase during March and April, while the highest value was obtained in June. Among animals living in the laboratory, no period of maximum or minimum values can be fixed for reasons which have been stated above. In one case (Group II) a clearly defined minimum occurred in March and this was succeeded by a definite rise; in other cases, the lowest values occurred in May and during the 1st week of June. And these were succeeded by a slight rise.

Differences such as those cited above may be of no particular importance. At any rate, there is no reason to assume that the course of events in man and animal should agree in all respects, or that variations in the calcium and inorganic phosphorus content of the blood of animals should follow a perfectly uniform course with respect to time. On the contrary, from what is known concerning analogous variations in organic constitution (5) and susceptibility to disease (6), it is to be expected that both the time of occurrence and the extent of any change in the chemical composition of the blood would vary from year to year. Moreover, it has been found that qualitative and quantitative differences will occur even among groups of animals that are under observation at the same time, so that one must reckon with variations in the response of animals, as well as with variations in the action of causative factors.

The point to be emphasized is that systematic or orderly variations in blood calcium and inorganic phosphorus do occur; in some instances,

it may be the calcium; in others, the phosphorus that is affected, or both substances may be affected in varying degrees. In the final analysis, however, it is the effect of these changes on the calcium-phosphorus equilibrium which concerns us most, and while various expressions of this relation may show greater or less change than either calcium or phosphorus, the relations do vary in much the same manner as the absolute amounts of the two substances.

Periodic variations in blood calcium and inorganic phosphorus have been referred to as seasonal variations. Hess and Lundagen attributed the increase and decrease of inorganic phosphorus in the blood of infants to changes in the available amount of ultra-violet radiation but they recognized the possibility that other factors might contribute to the production of these conditions. There is abundant evidence to show that ultra-violet rays exercise a profound influence on the inorganic phosphorus content of the blood of both man and animals, and that certain phases of calcium metabolism are likewise affected but analogous effects can be produced by other means (dietary).

The results obtained in these experiments could be explained in part as light effects. This is particularly true of the changes that occurred in inorganic phosphorus. On the assumption that the inorganic phosphorus in the blood is determined to a large extent by ultra-violet rays of short wave-length, one could account for the variations observed among animals living out of doors and for the progressive decrease that occurred when they were brought into the laboratory and thus deprived of this type of radiation. Among animals living in the laboratory, it would be difficult, however, to account for periodic increases in inorganic phosphorus or even variations in the rate of decrease as an effect of short wave-length ultra-violet radiation, and an explanation of the results obtained for calcium would be even more difficult.

Increasing age and the inactivity incident to cage life might also account for a part of the progressive changes in both calcium and inorganic phosphorus, but not for periodic variations. Moreover, as the rate of decrease in phosphorus, with respect to time, was decidedly irregular, it is not likely that age and cage life *per se* were decisive factors in the production of these changes.

Progressive changes in the chemical composition of the blood might be accounted for in various ways, but under the conditions of these experiments, no satisfactory explanation can be offered for the periodic variations. It is evident that the occurrence of these changes is referable to some condition of life which varies roughly with the seasons and is capable of exerting its influence on animals living indoors as well as out of doors, but not necessarily to an equal degree or in precisely the same manner. It is highly probable that ultra-violet rays, in a positive or negative way, played an important rôle in the production of the results reported, but it is practically certain that this was not the only factor concerned. Stated in a general way, it may be said that, while a number of factors probably contributed to the production of the variations observed, the indications are that the light environment, including visible as well as ultra-violet rays, played an important rôle.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of 5 groups of normal animals over periods of 4 to 8 months. The material included animals from recently acquired stocks as well as animals that had been living under laboratory conditions for long periods of time. The results were analyzed with especial reference to the occurrence of periodic and progressive variations in the absolute amounts of calcium and inorganic phosphorus and the relations between the two substances.

It was found that, among animals living in the laboratory, both calcium and inorganic phosphorus, as well as all expressions of the equilibrium between the two substances, exhibited a definite tendency to a progressive increase or decrease, as the case might be, and that clearly defined periodic variations occurred in all classes of animals. The progressive change was most marked in the case of inorganic phosphorus, the periodic change in the case of calcium, while both conditions were clearly shown by various expressions of the relation between calcium and inorganic phosphorus.

BIBLIOGRAPHY.

1. Brown, W. H., *J. Exp. Med.*, 1928, xlvii, 539.
2. Brown, W. H., *J. Exp. Med.*, 1928, xlvii, 563.
3. Hess, A. F., and Lundagen, M. A., *J. Am. Med. Assn.*, 1922, lxxix, 2210.
4. Grant, J. H. B., and Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 315.
5. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1926, xliv, 653.
6. Brown, W. H., Pearce, L., and Van Allen, C. M., *Tr. Assn. Am. Phys.*, 1924, xxxix, 351; *Proc. Soc. Exp. Biol. and Med.*, 1923, xx, 476; 1924, xxi, 371.

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THE MODE OF SPREAD OF A FRIEDLÄNDER BACILLUS-LIKE RESPIRATORY INFECTION OF MICE.

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PLATE 33.

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During observations of spontaneous *B. enteritidis* infection among special populations of mice (1, *a*), we noted the sudden appearance of a second disease which killed a great many of the animals. It arose first as an epidemic in late August, 1925, continuing in waves during the autumn and winter, and finally, in the spring, disappearing completely. A second similar outbreak occurred in August, 1926. The dead mice showed at autopsy bilateral pneumonia, and yielded in cultures from blood and lung an organism resembling Friedländer's bacillus.

The disease, in its sudden onset, high morbidity and mortality rates, its recurring outbreaks decreasing in severity, and its eventual disappearance, suggested somewhat human plague of the middle ages and the recent epidemic of influenza. For this reason, it was chosen for study, and its manifestations analyzed according to the experimental methods which we have employed in the investigation of mouse typhoid (1, *b*). The present paper describes the clinical course, pathology, and bacteriology of the disease, and records certain special studies of reactions between the bacteria and their host.

The literature on Friedländer bacillus-like infections, save for that relating to pneumonias of human beings, is scant and indefinite. Human case reports of Friedländer bacillus lesions of various organs, especially lesions situated near the upper respiratory tract, are numerous, however. Abel and Hallwachs (2) give references in which are described the recovery of this class of organisms from the atmosphere, earth, dust, water, and slime. Animal strains were reported by Pfeiffer in 1889 (guinea pigs (3)); Klein in 1889 and 1891 (guinea pigs and mice (4)); Weaver in 1897–99 (guinea pigs (5)); Skschivan in 1900, 1903 (rats (6)); Perkins in 1901 (guinea pigs (7)); Sachs in 1902 (rats (8)); Schilling in 1902 (rats

(9)); Toyama in 1903 (rats (10)); Aujeszky in 1904 (rats (11)), and Xylander in 1906 (rats (12)). Xylander refers also to communications on the subject by Fricke, Wilde, Fasching, and Gaffky. In reading these reports, however, one receives the impression that the organisms described form a rather heterogeneous group of capsulated bacteria, the effects on animals of which, were in many instances, quite different from that associated with typical strains of Friedländer's bacillus.

Since 1912, little has been written on Friedländer bacillus infection of animals, except for two articles by Holman (1916 (13)) and Branch (1927 (14)), in which the spontaneous disease in guinea pigs was described.

The general features of the disease in mice, as observed by us, are as follows:

Clinical Course.—The incubation period is about 48 hours. When a few carriers are placed in contact with groups of 100 or more mice, a certain number dies within 5 days, while 50–70 per cent are dead within 2 weeks. An occasional animal with signs and symptoms of pneumonia recovers, while a small number seems to resist infection altogether. Similarly, when a culture of the bacilli is introduced artificially into the nares of mice, a few animals die on the 2nd day, 70 per cent by the end of the 1st week, and a small number survives as nasal carriers or appears entirely refractory.

Pathology.—In mice dying of this infection, the autopsy shows subserous, petechial hemorrhages, such as occur in other septicemic infections, and extensive inflammation of the respiratory tract. The lungs are inflated, uniformly red and moist, and the serosa is covered with a seropurulent exudate. On section the lung tissue is red and wet, contains little air, and the exuded fluid is viscid and "stringy."

The microscopic lesions vary in extent and degree. In most instances there is dilatation of the interstitial capillaries, with or without accompanying interstitial hemorrhage and edema. Many alveoli contain serum and, occasionally, red blood cells. Beneath the pleura the congestion and edema are especially prominent. In many cases there is a fibrinous cellular exudate over the surface endothelium. The more advanced specimens show perivascular, interstitial, and intraalveolar accumulations of mononuclear and a few polymorphonuclear cells. Blood vessel walls are infiltrated and generally surrounded by cells; bronchi and tubules appear normal.

When groups of mice are given small doses of the organisms (200-600 intranasally), the pulmonary lesions described above are reproduced, and the differences observed are found to be related directly to the duration of life of the animals inoculated. For instance, mice dying 48 hours after the instillation show perivascular accumulations of large numbers of bacilli, perivascular round cell infiltration, and interstitial congestion; while those surviving 4 to 5 days show fewer bacteria, diffuse interstitial congestion and edema, and accumulations of leucocytes. After a longer period the alveoli are filled with leucocytes.

The pneumonic condition is in general similar to that described by Branch in spontaneous guinea pig infections (14) and by Stillman and Branch in experimental Friedländer infection in mice (15). The nature of the lesions studied by us suggests that the inoculated bacilli reach the lungs by way of the blood stream rather than by the bronchi (1, *d*).

Bacteriology.—The Friedländer bacillus-like organisms may be cultured from the nasal passages, lungs, and blood. Except in cases of severe septicemia, they are not found in the intestinal tract. Under dark-field illumination they appear as large, non-motile, homogeneous, blunt rods. They are Gram-negative; the capsules stain vividly,

TABLE I.

Cross-Agglutination Tests Made by Dr. Julianelle with His A, B, C, and X Friedländer Strains.

Strain	Friedländer type sera: dilution 1:5					Mouse Strain 1. Serum 1:5
	Type A	Type B	Type C	Group X I	Group X II	
Mouse Strain 1	−	−	−	−	−	+++
" " 2	−	−	−	−	−	+++
" " 3	−	−	−	−	−	+++
" " 4	−	−	−	−	−	+++
" " 5	−	−	−	−	−	+++
Friedländer Bacillus "A"	+++	−	−	−	−	−
" " "B"	−	+++	−	−	−	−
" " "C"	−	−	+++	−	−	−
Group X I	−	−	−	+++	−	−
" X II	−	−	−	−	+++	−

and Wright's blood stain exhibits a large enveloping material surrounding a deeper staining substance. Dextrose, maltose, xylose, salicin, and mannite are fermented in 24 hours; saccharose in 3 to 4 days, and lactose in 7 to 12 days. Milk is acidified; no indol is formed; nitrates are reduced to nitrites.

The serologic reactions of the strains obtained by us are practically identical. Sera obtained from rabbits by injecting dead and living cultures agglutinated all strains to a maximum titer of 1:640. No antigenic relationship was established with other known strains of Friedländer's bacillus.

A series of cross-agglutinations and thread tests with five of our mouse strains and with Friedländer's bacilli, Type A, B, C, and X, was made for us by Dr. Julianelle (16). Table I gives the results. The five type sera of Friedländer's bacillus failed to agglutinate any of the mouse strains, and the mouse strain serum agglutinated none of Julianelle's five type strains of Friedländer's bacillus.

Colonies of the bacteria growing on agar plates from direct mouse autopsy culture are large, moist, and viscid, "stringing" easily on the platinum loop (Figs. 1 and 2). Bacilli from young, single cell colonies show no mucous material. At a later period, the entire colony, save for the peripheral zone of activity dividing bacteria, is composed of the enlarged cells, which in mass present a mucoid appearance.

Growth requirements of these organisms are relatively simple. No enriching substances, carbohydrates, serum, "X", or vitamine factors need be added to plain infusion agar or broth, pH 7.0–7.4. However, considerable sensitivity to temperature changes exists.

Experiment 1 compares the growth of cultures at 37° and 23°C.

Two series of tubes containing 5 cc. of plain infusion broth of pH 7.4 were inoculated with different dilutions, 10^{-1} to 10^{-9}, of a 48 hour broth culture of Mouse Strain 3. One series was incubated at 37°; the other left at room temperature, 23°. Growth in the two series was compared at 24 and 48 hours. 3 days later the cultures were examined by dark-field illumination, by staining methods, and by replating. 6 days after inoculation, the numbers of viable bacteria in each tube of the series were counted by the plating method.

The first four tubes of the 37° series showed good growth at 24 hours; the remaining five, 10^{-5} to 10^{-9} inclusive, were sterile. The entire series grown at 22° were turbid at 24 hours. The original culture used for inoculation contained about 800,000,000 organisms. Hence, as the findings show, an inoculation of 100,000 organisms was required to produce growth at 37°, whereas the smallest number, less than 10, grew at 22°. No further growth occurred at 48 hours. At this time, examination of cultures by dark-field and staining methods showed autolysis, great swelling, and reduction in numbers of bacilli in the tubes of the 37° series; the 22° cultures appeared normal. Counts at 6 days showed less than 100 bacilli per tube in the first four dilutions of the 37° series; the rest were sterile. Plates of tubes of the 22° series averaged about 170,000,000 organisms per cc. per tube.

The tests were repeated with other strains with essentially the same results. Hence, the optimal growth temperature is considerably less than 37°.

The next experiment was planned to ascertain the duration of life of these organisms in fluid culture.

Experiment 2.—Four flasks with 300 cc. of broth in each were inoculated with a 48 hour culture of Strain 1. Two received about 12,000 organisms and two about 10; actual counts were made immediately. One flask of each dilution was then incubated at 37°; the two others were left at room temperature, 22°. Counts were made of the numbers of bacteria in each flask at 3, 6, 25, and 52 hours, and at 10 months. The results are shown in Table II.

TABLE II.

Growth of Small Numbers of Friedländer-Like Bacilli at 37° and 23°C.

Time after inoculation	No. bacteria per cc.			
	Flask 1: inoculation 10^{-3} 22°	Flask 2: inoculation 10^{-3} 37°	Flask 3: inoculation 10^{-5} 22°	Flask 4: inoculation 10^{-5} 37°
hrs.				
0	12,000	15,000	10	10
3	48,000	9,000	0	0
6	70,000	3,000	30	0
25	180,000,000	0	18,000,000	0
52	325,000,000	0	280,000,000	0
mos.				
10	6,000,000*	0	5,000,000*	

* Rough variants 100 per cent. No mucoid forms.

Both incubated (37°) flasks were sterile at 24 hours and remained so. The two flasks standing at 22°, room temperature, showed good growth at 25 hours and viable organisms 10 months later. At this time, however, the mucoid colonies were replaced entirely by the rough variant colony type. Hence these cultures are found to be relatively simple in their requirements and able to survive for long periods of time at temperatures of about 20–24°C. Higher temperatures, however, prove harmful.

Colony type variation occurs readily with the Friedländer group of bacilli. Hadley and Julianelle have summarized the reports of early investigators. Hadley (17) found that rough colonies of Friedländer bacillus arise when cultures are grown for some days in broth or agar. Branch witnessed the same phenomenon in the cultures from guinea pigs (14). Julianelle noted transformations in his A, B, and

C type strains grown in antiserum (16). Our mouse cultures lose the mucoid colony appearance when grown in antiserum, and when kept 2 or more weeks in broth or agar. The variant colonies are non-mucoid, quite small, opaque, with regular margins and granular surfaces (Figs. 1, 2, 3).

The variant mouse strain colonies are not found under natural conditions. During the past 2½ years, we have cultured the nasal passages of more than 2000 mice and autopsied at least as many more, and obtained only mucoid colony forms. We conclude, therefore, that the variant types do not occur in recognizable numbers during either the interepidemic or epidemic phase of the native mouse disease.

Single cell cultures from the variant colonies are relatively stabile in spite of various manipulations *in vitro* and *in vivo*, a reverse transformation from variant to the original mucoid form was not accomplished.

Bacilli from the variant colonies are smaller than those from the mucoid colonies, and lack the enveloping material; otherwise the two resemble each other. The short rods are Gram-negative and non-motile, fermenting the characteristic sugars, acidifying milk, and reducing nitrates to nitrites. They agglutinate in antimucoid sera to more than double titer. They are not flocculated by sera from Julianelle's Type A, B, and C strains.

Microbic Virulence and Host Susceptibility.

We have determined the effects of different numbers of bacilli on the amount and severity of the experimental infection, and have made titrations of the virulence of the same and different bacterial strains, over a period of more than 2 years.

Technique.—The general principles governing the technical procedures of these titrations have been described elsewhere (1, *b*). Briefly, we consider it essential to reproduce the natural conditions of infection as nearly as possible. The dosage, virulence, and host susceptibility tests are planned in such a way as to remove as far as possible disturbing variables. Thus, under given conditions, relatively constant results are secured. The circumstances most nearly reproducing the native infection are adopted as a standard. This

standard is then used for comparison for titrations of dosage, virulence, and host factors.

Cultures are grown in a uniform medium and treated alike throughout. Dosage is estimated in numbers of bacilli administered. The bacilli, suspended in a drop of salt solution, are introduced through a small glass pipette into the nasal passages of each mouse. The spasmodic inhalations of the animal aid the taking in of the fluid. Mice from the inbred Rockefeller Institute stock, raised under uniform environmental conditions, about 12 weeks of age, weighing 18-20 gm., and not previously exposed to the infection are used (1, *c*). The inoculated mice are placed in separate cages.

Standard Curve.—At the outset, the precision of the titration technique was determined by performing a number of titrations in duplicate and observing the amount of variation in the end results.

Technique.—Strain 1, obtained on October 25, 1926, from a mouse in one of the special populations, was used throughout. For each titration, a subculture in broth was made from the stock slant and grown for 48 hours at 23°. A dose of 200–600 bacteria per mouse was found to represent natural conditions most satisfactorily. Hence each animal was given intranasally, by glass capillary tube, 1 drop of a 1:10,000 0.85 per cent salt dilution. This volume contained 200–600 organisms, as checked each time by dilution plates. The mice were chosen according to the above specifications. After inoculation, each animal was numbered and placed in a separate jar. The entire number was then divided arbitrarily into groups of 20, 25, 50, or 100, according to the amount used, and the mortality in each group was compared. Dead mice were autopsied and cultured. The results of these tests are summarized in Table III.

On November 16, 1926, a titration was made with 50 mice, divided into two groups of twenty-five. Each group showed close agreement in amount and rate of death. 84 per cent were dead in each on the 18th day; none died thereafter (Table III). On November 22, a similar test was made, resulting in a final mortality on the 42nd day of 86 per cent, with a variation between groups of 6 per cent. A third titration on December 2 with three different doses and six groups of twenty mice each showed total mortalities of 92.5 per cent with a group variation of 2.5 per cent (1250 bacteria per mouse), 90 per cent total and group mortality (380 bacteria per mouse), and 55 per cent, with a variation between groups of 10 per cent (58 bacteria per mouse). On December 16, a fourth test with two groups of twenty and a dose of 195 bacilli resulted in the death of 70 per cent total and 5 per cent group variation mortality. A fifth titration on December 20, with two groups of twenty-five and a dose of 960 bacteria per mouse, showed a final mortality of 90 per cent with a 2 per cent variation of group mortality. On January 5, 1927, two groups of 50 each received 15,000 organisms per mouse, and two groups of 50 a dose of 115 bacilli. The mortalities at 42 days were 86 per

TABLE III.

Mortality during Days after Inoculation.

			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	
Date	*Dose*	*Group*																							
11–16–26	250±	1– 25	2	4	3	8			2		2														21
		26– 50		6	8	3		1	1		2														21
		Total	2	10	11	11		1	3		4														42
11–22–26	350±	1– 25		8	3	2	2			2				1		1							1		20
		26– 50	1	10	3	4	2								2	1									23
		Total	1	18	6	6	4			2				1	2	2							1		43
12– 2–26	1250	1– 20	3	10	1	2		1		1								1							19
		21– 40		12	2			1	1			1	1												18
		Total	3	22	3	2		2	1	1		1	1					1							37
	380	1– 20	4	5	2	1			1	5															18
		21– 40	2	5	4	2	2		1	2															18
		Total	6	10	6	3	2		2	7															36
	58	1– 20		1	5			1		2															9
		21– 40		2	4	3			1	2							1								13
		Total		3	9	3		1	1	4							1								22
12–16–26	195	1– 20		4	5	1				1			1	1											13
		21– 40	1	5	6					1	2														15
		Total	1	9	11	1				2	2		1	1											28

12–20–26	960	1– 25	1	12	8	1																			22
		26– 50	2	9	9	2	1																		23
		Total	3	21	17	3	1																		45
1– 5–27	15,000	1– 50	1	25	12	2	2	1						1	1										45
		51–100		27	11	1		1						1											41
		Total	1	52	23	3	2	2						2	1										86
	115	1– 50		11	4							2	1		6										24
		51–100		12	4				1		1	2			10	3									33
		Total		23	8			1	1		1	4	1		16	3									57
1– 7–27	187	1– 50		23	13	2	1		2					1											42
		51–100		22	11	1	1		1					1											37
		Total		45	24	3	2		3					2											79

cent with a 4 per cent and 57 per cent with a 9 per cent final difference in group mortality.

The results of duplicate titrations, of which the above are a part, showed that the experimental technical error was insignificant; hence tests carried out as described above proved sufficiently accurate.[1] Furthermore, the mortality rate per day was found to be so uniform that average figures were taken to represent numerically the reaction of the Rockefeller Institute strain of mice to a dose of 200–800 bacteria of Strain 1. These total mortality percentages, when plotted against time in days, formed a frequency curve similar to those obtained in mouse typhoid studies (1, *b*) (Text-fig. 1). Hence, measurement of variations in dosage, virulence, and host susceptibility were compared

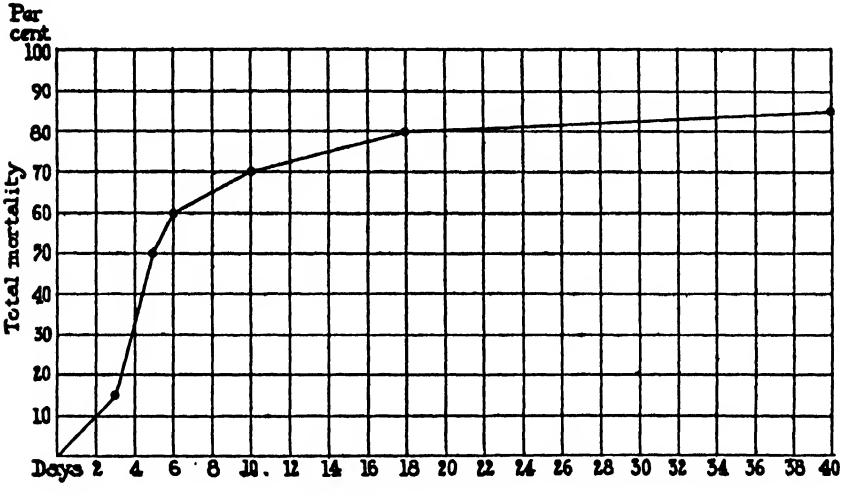
[1] Dr. John W. Gowen analyzed the data contained in Table III and commented upon it as follows: "Mathematically analyzed, using mortality rates and weighing them equally, the variation within the groups is quite insignificant in contrast to the variation between the groups made at different times, indicating that the technique has been satisfactory, thus:

Variance	Degrees of freedom	Mean square
Between groups	9	375.1 Z = .927 where for $P = .05$ Z = .56
Within groups	10	58.7

"Two possible causes of the significant variation between groups are obvious in the data. The first is variation in dosage, as indicated by the plate counts, and the second is seasonal variation, although the controlled environment of the mice and cultures make such an interpretation doubtful. The data are not well suited to attempts to separate these possibilities since the number of repeated titrations at different dates is not sufficient. However, if the material be drawn as a graph with death rate as one coordinate and dosage of bacteria as the other, it is noticed that for dosages from 58–400 there is a rapid almost linear rise in death rate from 55–390. At 400 bacteria the death rate becomes essentially the same to dosages up to 15,000. This suggests dosage as the important cause of the variation between groups. Approaching the problem slightly differently, there are two dates on which groups of mice were given different dosages, December 2, 1926, and January 5, 1927. Analyzing this data for influence of date of inoculation, the mean variance between dates is found to be 70 and within dates 433. Clearly the date of inoculation played an insignificant part in the death rate. As the cause of variation within the dates is the dosage, we may attribute to it the major rôle in causing the variation in the death rates within these data with influence of season or variation in technique as but minor and insignificant causes of variation."

either with these average figures or with those obtained from a control group tested simultaneously.

Dosage.—Observations of the spontaneous disease among the mice of the special populations indicated that contact with a very small number of the bacilli is fatal to most individuals. For example, surviving carriers in the mouse populations were found to harbor in their nasal passages but very few of the Friedländer bacilli, while mice from which fifty or more colonies were cultured invariably died within a few days. These findings were confirmed by actual titrations which showed



TEXT-FIG. 1.

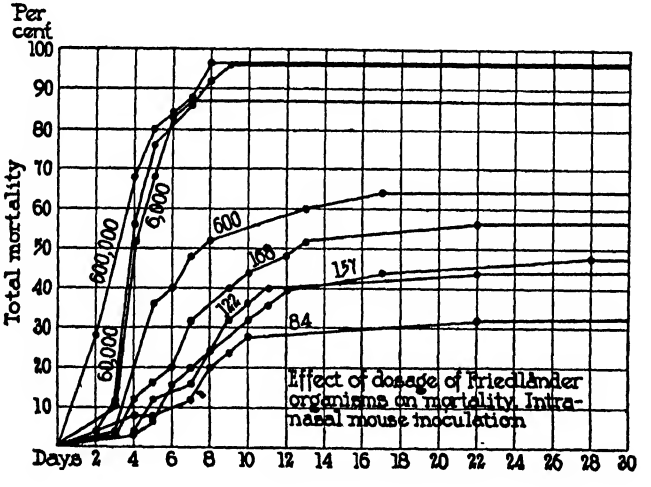
that while a small number of mice survived even a maximal dose, as few as 500 bacilli per mouse killed about 80 per cent within a week. Tests were made to determine this relationship more accurately.

Experiment 3.—Strain 1 was transferred from the stock slant to meat infusion broth, pH 7.4, and left at room temperature (23°) for 48 hours. Dilutions were then made in 0.85 per cent NaCl, counted by the plate method, and given intranasally to mice by means of a coarse capillary pipette. Eight dilutions were used; each was given to twenty-five mice. After inocuation, the animals were placed in separate jars. Mortality records were kept and autopsies done on all dead animals.

The duration of life of the individuals of each group is plotted in Text-fig. 2. The mortality rates of groups receiving 6,000, 60,000, and

600,000 bacilli were quite similar. No deaths occurred after the 9th day; one animal of the 600,000 and 60,000, and three of the 6000 group survived 30 days. In the remaining groups death rate and total mortality were related closely to dosage. 64 per cent of the group given 600 bacteria succumbed, 56 per cent of those given 168, 44 per cent of those given 122, and 32 per cent of those receiving 84.

These tests show that small doses (less than 100 bacilli), given to a group of twenty-five mice, are fatal to certain individuals; that doses ranging from this to over 1000 lead to a progressively increasing death rate and total mortality in groups of similar size; and that doses larger



TEXT-FIG. 2.

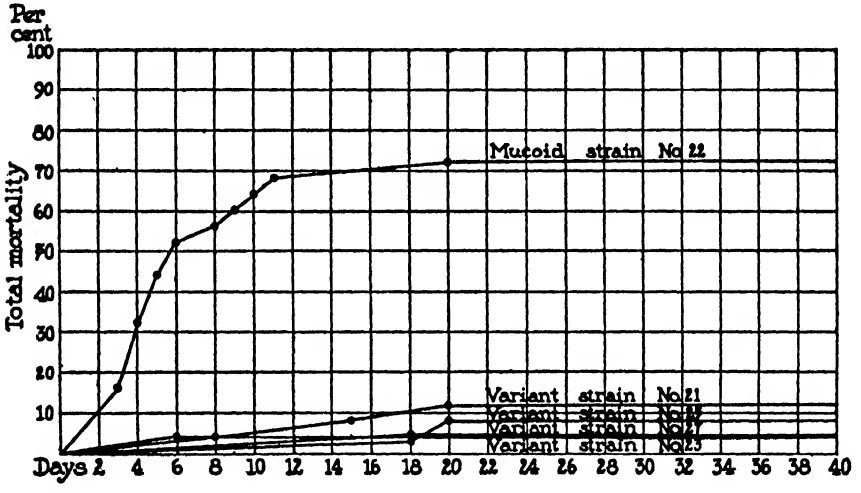
than 6000 organisms do not increase the number of deaths beyond a certain point. Regardless of the size of the dose, some individuals survive.

Virulence.—The virulence of the bacilli has been determined by tests similar to those described above. The standard dose of about 600 organisms was instilled intranasally. Sufficient animals were used to run each titration in duplicate. Strains of unknown pathogenicity were compared directly with known cultures, or indirectly by means of the standard curve described above (Text-fig. 1).

Repeated titrations of single strains over a period of 12 months showed no significant changes in virulence. The results of the tests

on Strain 1 are given in Table III. This culture has been kept on agar at 4°C. and transferred every 4 weeks. It was obtained from a mouse of a known susceptible race (1, *c*), 2 days before the onset of a severe spontaneous epidemic. The degree of virulence of the type-pure culture was found to remain relatively constant. Three other strains kept type-pure and tested in the same way showed insignificant fluctuations in virulence.

Rough variants obtained from old broth or agar cultures proved far less virulent than the parent mucoid type. The following test illustrates this difference.



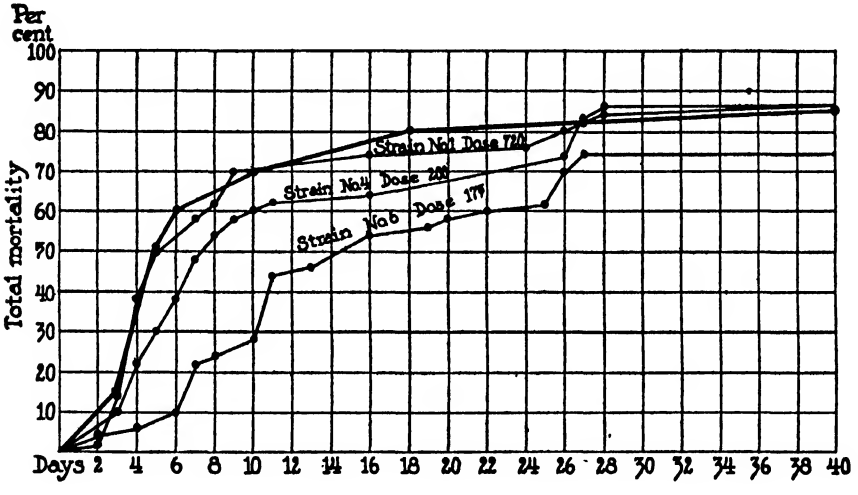
TEXT-FIG. 3.

A mucoid type strain, No. 22, obtained from a mouse of one of the special populations, September, 1925, its rough variant, No. 23, and three other rough variants from mucoid type strains isolated at about the same time were grown 48 hours in broth at 22°C., diluted 1:10,000, and administered intranasally to mice. Each culture was given to twenty-five individuals. The mortality record is plotted in Text-fig. 3.

The mucoid culture, No. 22, showed high virulence characteristic of the type, while its rough variant, No. 23, together with the three other rough strains, Nos. 21, 25, and 27, killed only very few of the animals—4.4, 12, 8, and 4.4 per cent respectively.

A number of titrations were made of cultures taken from mice of the

special populations in which the spontaneous infection was prevalent in endemic and epidemic form. The strains were chosen under varying conditions, with a view to determining differences in lethal power coincident with high or low population mortality, with cultivation of the bacilli in the circulating blood of susceptible individuals, and in the nasal passages of animals resisting infection. A chart describing the course of the infection among the special populations, with a record of the days on which these cultures were obtained, will be published later; at present it will suffice to state briefly the amount of disease prevailing at the time each culture was obtained.



TEXT-FIG. 4.

The method of titration of virulence described in a previous paragraph was employed throughout. Dilutions were made to insure a dosage of about 600 bacilli per mouse. Due to slight irregularities of growth, however, the actual counts as determined by plating varied considerably from this number. Such fluctuation in the relatively small doses of these organisms given has been shown to affect death rate considerably (Experiment 3), and it is responsible, we believe, for the irregulatities in some of the virulence curves.

The results of the virulence trials with unknown strains have been compared either with the standard control curve for Strain 1, or with a simultaneous control titration with Culture 1. Each group of mice

TABLE IV.

Mortality during Days after Inoculation.

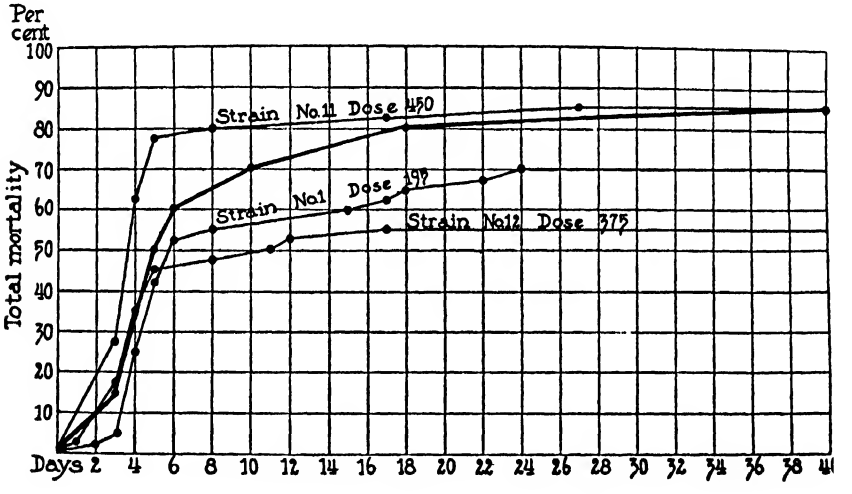
Date	Dose	Group	0–2	2	4	6	8	10	12	14	16	18	20	22	24	26	28–30	Total	Death rate	Average age at death
11–22–26	720	1– 25			8	3	2	2			2				1		1	19	76	9.95± 1.07
Strain 1		26– 50		1	10	3	4	2								2	1	23	92	9.35± 1.04
		Total		1	18	6	6	4			2				1	2	2	42	84	9.61± .75
	200	1– 25			8	4	4	1	1		1					2	2	23	92	11.17± 1.16
Strain 4		26– 50			3	4	4	2								3	4	20	80	14.90± 1.49
		Total			11	8	8	3	1		1					5	6	43	86	12.91± .95
	175	1– 25				1	5		4	1	2		2			3	2	20	80	16.70± 1.12
Strain 6		26– 50		2	1	1	2	2	4		2			1		2		17	68	13.00± 1.17
		Total		2	1	2	7	2	8	1	4		2	1		5	2	37	74	15.00± .83
12–16–26	450	1– 20			15	2	1											18	90	5.44± .17
Strain 11		21– 40			10	4						1					1	16	80	7.88± 1.08
		Total			25	6	1					1					1	34	85	6.59± .53
	195	1– 20			4	5	1				1			1	1			13	65	9.92± 1.26
Strain 1		21– 40		1	5	6					1	2						15	75	8.33± .90
		Total		1	9	11	1				2	2		1	1			28	70	9.07± .76
	375	1– 20		1	7	2			2			1						13	65	7.46± .83
Strain 12		21– 40			6	2	1											9	45	5.89± .31
		Total		1	13	4	1		2			1						22	55	6.82± .52
12–20–26	960	1– 25		1	12	8	1											22	88	5.82± .19
Strain 1		26– 50		2	9	9	2	1										23	92	6.22± .26
		Total		3	21	17	3	1										45	90	6.02± .16
	225	1– 25			6	11	4			1								22	88	7.18± .31
Strain 13		26– 50			7	5	6											18	72	6.89± .27
		Total			13	16	10			1								40	80	7.05± .21
	580	1– 25			9	5	1	1	1	1								18	72	7.11± .47
Strain 15		26– 50			11	5		1									1	18	72	7.22± .87
		Total			20	10	1	2	1	1							1	36	72	7.17± .49
1– 7–27	150	1– 25		1	6	7	2	8										24	96	7.83± .36
Strain 17		26– 50			6	11	3	2										22	88	7.09± .25
		Total		1	12	18	5	10										46	92	7.48± .22

TABLE IV—*Concluded.*

Date	Dose	Group	0–2	2	4	6	8	10	12	14	16	18	20	22	24	26	28–30	Total	Death rate	Average age at death
1-7-27	187	1- 50			23	13	2	1	2					1				42	84	6.76± .34
Strain 1		51-100			22	11	1	1	1					1				37	74	6.57± .36
		Total			45	24	3	2	3					2				79	79	6.67± .25
	110	1- 25			9	5	1							1	1			17	68	8.06± .97
Strain 16		26- 50			12	5	1			1								19	76	6.26± .37
		Total			21	10	2			1				1	1			36	72	7.11± .51
1-19-27	400	1- 50			14	17	6	3	1					1				42	84	7.43± .33
Strain 18		51-100			3	8	4	6	4	2				1				28	56	9.93± .49
		Total			17	25	10	9	5	2				2				70	70	8.43± .29
	630	1- 50			7	9	7	4	3						1			31	62	8.68± .47
Strain 19		51-100			5	19	3	4	1	1				1				34	68	8.24± .40
		Total			12	28	10	8	4	1				1	1			65	65	8.45± .31
2-28-27	500	1- 25			10	9		1		1								21	84	6.62± .35
Strain 34		26- 50			12	8												20	80	5.80± .15
		Total			22	17		1		1								41	82	6.22± .20
	590	1- 25			9	8	2	1										20	80	6.50± .25
Strain 19		26- 50			9	7	3					1	1					21	84	7.67± .62
		Total			18	15	5	1				1	1					41	82	7.10± .35
	320	1- 25			13	3	3											19	76	5.95± .23
Strain 33		26- 50			10	5	1	1	1		1							19	76	7.11± .50
		Total			23	8	4	1	1		1							38	76	6.53± .28
	540	1- 25			12	3	1	1				1						18	72	6.67± .54
Strain 32		26- 50			9	5	2	1										17	68	6.41± .29
		Total			21	8	3	2				1						35	70	6.54± .31
5- 2-27	420	1- 25			13	8	1											22	88	5.91± .17
Strain 50		26- 50		1	8	6	3											18	72	6.22± .26
		Total		1	21	14	4											40	80	6.05± .15

receiving a given culture was divided arbitrarily into two subgroups of equal numbers, and the mortality of each compared with that of the total group. Hence a mean daily mortality figure was obtained and the extent of deviation from this mean of each subgroup noted.

Test 1. The Virulence of Freshly Isolated and Stock "Epidemic" Strains.— Strain 1 was obtained from the heart's blood of a susceptible Lathrop mouse, October 15, 1926, 2 days before the outbreak of a severe epidemic; Strain 4 from a similar animal, November 17, 1926, just prior to a similar wave. Strain 6 was isolated at the peak of an epidemic, August, 1925, from an individual of a different, though similar population. Since that time, it has been kept on plain agar at 4°C. On November 22, 1926, each strain was given to 50 mice which were then numbered and placed in separate jars. Two groups of twenty-five mice each were made arbitrarily for comparison of results. The dosage employed was as follows Strain 1, 720 organisms per mouse; Strain 4, 200 organisms per mouse; Strain 6 175 organisms per mouse. The results are summarized in Text-fig. 4 and Table IV

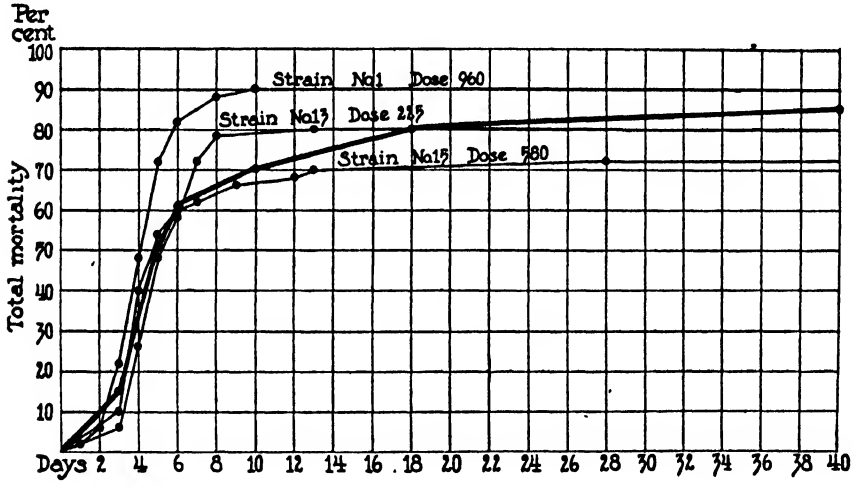


TEXT-FIG. 5.

The mortality of the groups receiving Strains 1 and 4 was similar throughout the period of the experiment. 84 and 86 per cent respectively, were dead on the 40th day. The final deviation from the mean of the subgroups was 8 and 6 per cent, and the average daily deviation 7.4 per cent for mice receiving Strain 1 and 8.6 per cent for those receiving Strain 4. The death rate of the group receiving Strain 6 was lower during the 1st week but deaths continued over a longer period of time. The final mortality was 74 per cent, with a deviation of subgroups from the mean of 6 per cent and an average daily deviation of 1.5 per cent. The mortality curves of the groups receiving Cultures 1 and 4 parallelled closely the standard mortality curve.

The two cultures, therefore, may be considered to be of equal virulence. Culture 6 seemed somewhat less effective, but this discrepancy is believed to be due to differences in actual dosage of bacilli received, rather than to any difference in their degree of virulence.

Test 2. The Virulence of Freshly Isolated "Epidemic" Strains Taken from the Nasal Passages of Healthy Mice.—Three strains were used in this test,—Strain 11 was obtained from the nasal passages of a healthy mouse of the so called "Friedländer" population 1 day prior to the outbreak of a severe epidemic, and Strain 12 from a similar mouse in the same population 1 week before the same outbreak. Strain 1 was again employed as control. Each culture was instilled into forty mice. The dose of Strain 1 proved to be 195 organisms per mouse, of Strain 11, 450, and Strain 12, 375. The results are shown in Text-fig. 5 and Table IV.

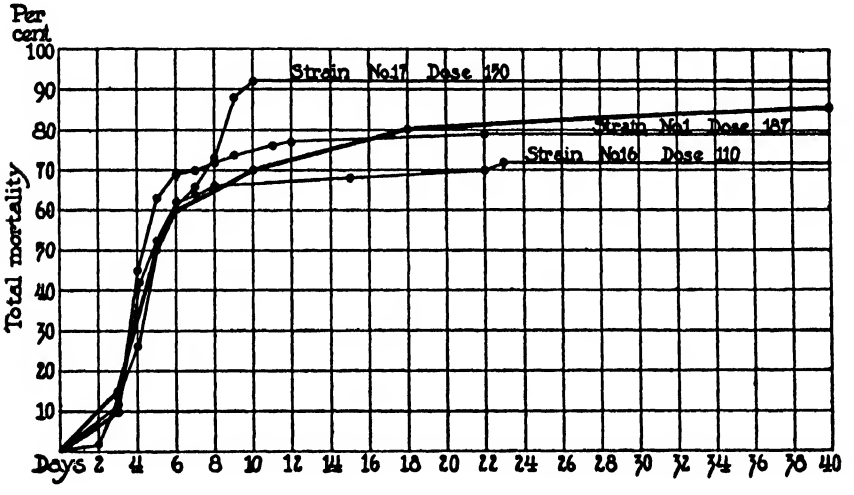


TEXT-FIG. 6.

The final mortality of the group receiving Strain 11 was 85 per cent with a deviation of the subgroups from the mean of 5 per cent and an average daily deviation of 7.4 per cent; that of the group receiving Strain 12 was 55 per cent, with a variation between groups of 10 per cent on the final day, and an average daily deviation of 4.9 per cent, while the final mortality of Strain 1 group was 70 per cent, with a 5 per cent variation and an average daily deviation of 5.6 per cent. The apparent low activity of Strain 12 was due, we believe, to undetermined differences in actual dosage received and not to a difference in virulence.

Test 3. The Virulence of "Epidemic" Strains Taken from the Nasal Passages of Surviving Mice.—Strains 13 and 15 came from individuals of the same population as Strains 11 and 12, and were obtained at the height of the epidemic mentioned above. The mice appeared healthy at the time the cultures were taken and survived the epidemic. Strain 1 was run at the same time as a control. Each culture was given to 50 mice. The dose of Strain 1 proved to be 960 organisms per mouse, of Strain 13, 225, and of Strain 15, 580. The results are summarized in Text-fig. 6 and Table IV.

The mortalities in all three groups were similar and approximated closely the figures of the standard mortality curve. 90 per cent of the Strain 1 group were dead on the 40th day, with the subgroups showing



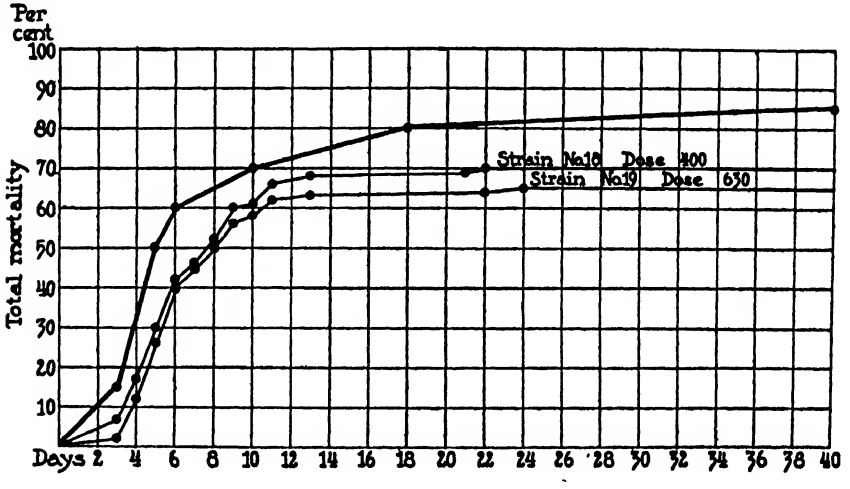
TEXT-FIG. 7.

a final deviation of 2 per cent and an average daily deviation of 2.2 per cent. The mortality of the group receiving Strain 13 was 80 per cent, subgroup variation 8 per cent, and an average daily deviation of 7.2 per cent, and that of the group given the No. 15 culture was 72 per cent, with subgroups identical at the end of the test and showing an average daily deviation of 1.3 per cent. The three cultures tested, therefore, may be considered as equally virulent.

Test 4. The Virulence of "Epidemic" Strains Taken from Heart's Blood of Infected Individuals.—Strain 16 came from a susceptible mouse of one of the special populations designated "G" population, at the height of a severe and long epidemic. Strain 17 came from a mouse of another special population designated

"Friedländer" population, also during the plateau of a severe and long spontaneous epidemic wave. Strain 1 was run simultaneously as a control. One hundred and eighty-seven bacilli of Strain 1 were given to each of 100 mice, 110 of Strain 16 to each of 50 mice, and 150 of Strain 17 to each of 50 mice. The results are shown graphically in Text-fig. 7 and Table IV.

The mortality curves of all three series are similar and follow quite closely the standard control curve. The final mortality of the Strain 1 group was 79 per cent, with a subgroup variation of 5 per cent and an average daily deviation of 4.3 per cent; of the Strain 16 group, 72 per cent, subgroup difference 4 per cent, and average daily deviation 5.2



TEXT-FIG. 8.

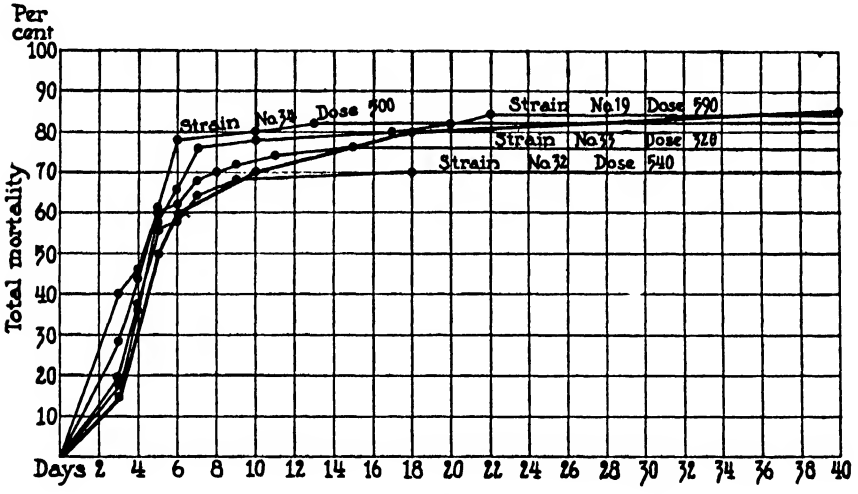
per cent, and of the Strain 17 group 92 per cent, subgroup variation 4 per cent, and average daily deviation 4.2 per cent. The three cultures therefore may be considered to possess equal virulence.

Test 5. The Virulence of "Epidemic" Strains from the Nasal Passages and Circulating Blood.—Strain 18 was obtained from the nasal passages of a healthy mouse of the special "G" population at the height of a severe epidemic. The individual from which this culture was taken survived. Strain 19 came from the blood of a mouse of "No. 6" population, dying at the height of a severe epidemic wave. Each culture was administered to 100 mice in doses of 400 and 630 organisms per mouse respectively. The results are charted in Text-fig. 8 and Table IV.

The mortality of each group was almost identical, and differed from that of the standard control curve by a small margin. The final

mortality of the No. 18 group was 70 per cent, subgroup difference, 14 per cent, average daily deviation 14 per cent; that of the No. 19 group was 65 per cent, with a subgroup variation of 3 per cent, and an average daily deviation of 2.9 per cent. For the instance of the large deviations in subgroups from the mean in the case of the No. 18 group, which is unique, we offer no present explanation. Nevertheless, these cultures may be considered equal to each other and to agree with Strain 1 in virulence.

Test 6. The Virulence of "Epidemic" and "Surviving" Strains.—Strain 19 was described in Test 5. Strains 32 and 33 came from the lungs of two mice of the



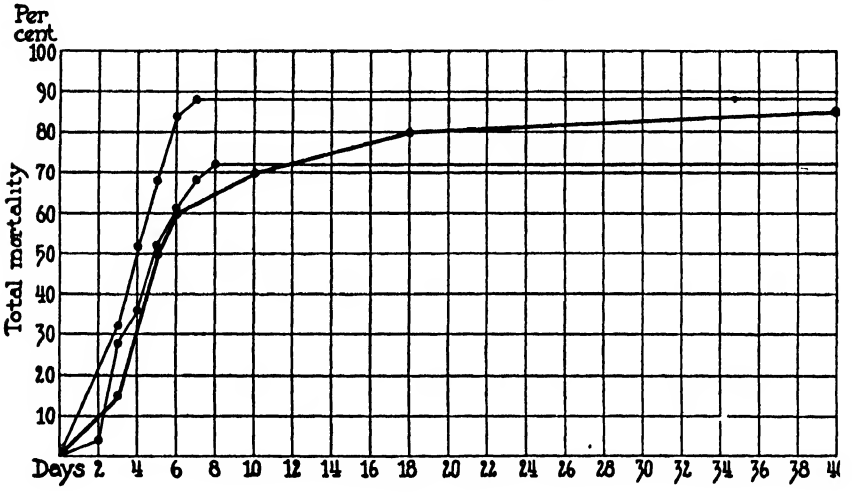
TEXT-FIG. 9.

"No. 6" population dying at the peak of a severe epidemic. Strain 34 came from the nasal passages of an individual of the same population, which had lived throughout the entire epidemic and was surviving and healthy in the interepidemic period at the time the culture was taken. Each strain was administered to 50 mice,—590 organisms per mouse of No. 19, 540 of No. 32, 320 of No. 33, and 500 of No. 34, the results of which are shown in Text-fig. 9 and Table IV.

The mortality curves resemble one another and the standard control curve of Strain 1. 82 per cent of the No. 19 group were dead on the 40th day. The final deviation of the subgroup was 4 per cent, and the average deviation 2.7 per cent per day. The final mortality of the No. 32 group was 70 per cent, with a final subgroup deviation of 2 per

cent and an average daily deviation of 1.5 per cent. That of the No. 33 group was 76 per cent, with no final difference in the subgroups and an average daily deviation of only 1 per cent; that of the No. 34 group was 82 per cent, with a final and average daily deviation of 2 per cent. These strains and Strain 1, then, are considered to be of the same virulence.

Test 7. The Virulence of a Surviving Culture, Strain 35.—This strain was obtained from the only carrier of the "No. 10" population, surviving a severe epidemic wave which had occurred 2 months previously. Within a week after the culture had been obtained from this mouse, it ceased to yield the Friedländer



TEXT-FIG. 10.

bacilli and at about the same time the disease disappeared entirely from the population. Two groups of twenty-five mice each were given the culture in a dose of 420 organisms per mouse. The results are shown in Text-fig. 10 and Table IV.

The mortality of each group is seen to approximate closely the figures obtained from the standard control curve. This culture, therefore, is considered to be of the same virulence as Strain 1 and all cultures previously tested.

The precision of the virulence titrations is ascribed to the fact that the experimental conditions were arranged so as to eliminate as many disturbing, unknown factors as possible, and to reproduce as nearly as possible natural conditions of infection. Hence the conclusion

seems justified that the virulence of type-pure mouse strains of Friedländer-like bacilli, with which we have dealt, remains constant under natural conditions.[2] Mucoid strains kept type-pure on agar do not

[2] Dr. Gowen analyzed the results of these virulence titrations and commented upon them as follows: "The results of Tests 1 to 7 inclusive may be analyzed for the influence of period in the epidemic at which the cultures were isolated in relation to death rate. This analysis is complicated by the effect of variation in dosage. There are at least two culture tests made for each group which will give some appreciation of this factor, however. Cultures before peak of epidemic were 1, 4, 11, and 12; at peak 6, 13, 15, 16, 17, 18, 19, 32, and 33, and in the inter-epidemic period were 34 and 50. The material is set out below:

Variance	Degrees of freedom	Sum of square	Mean square	
Between tests	18	2990	166.1	
Within tests	19	1384	72.8	Z = .412 where for P = .05 Z = .43
Total.....	37	4374		

"The variation between tests is greater than that within tests, as in the previous results for Strain 1. The significance of this difference is questionable, however. Z = but .41 while Z for P = .05 = .43. Furthermore, the variation between tests does not show the consistent relation when plotted against dosage of bacteria, that was previously shown for Strain 1. In fact, the death rates appear to be quite random so far as dosage is concerned.

"Two of the 18 degrees of freedom between tests are due to the point in the epidemic where the bacilli were isolated as indicated above. Dividing the data thus the variance and its associated constants are:

Variance	Degrees of freedom	Sum of square	Mean square	
Between epidemic points	2	150	75.0	
Within epidemic points	16	2840	177.5	Z = .431 where for P = .05 Z = 1.48
Total.....	18	2990		

"The analysis of the data on the epidemic point of bacterial isolation shows that the variation between epidemic points is much less than that within the different groups. The conclusion is consequently justified that the epidemic point of isolation of the bacteria plays no part in the total fatality of the epidemic it initiates in the Friedländer disease of mice. The differences within and between epidemic point groups are not significantly differentiated since Z = .43 while for P = .05 Z must be at least 1.48.

"The rapidity with which the organism kills for those animals dying offers another measure of the possible effect of period in the epidemic during which the organism was isolated. For the three groups as utilized above the average duration of life for those dying was for the preepidemic peak 8.14 ± .26;* peak of epi-

* Because of the skewness of the distributions these probable errors lose much of their meaning and must be interpreted with caution.

change in pathogenicity; strains obtained from animals before, during, and after epidemic periods, and in interepidemic times all agree in pathogenic activity, and strains taken from healthy carriers, from individuals acutely ill, and from animals dying of pneumonia and septicemia, are of the same high virulence. On the other hand, rough strains, developed in the laboratory, exhibit much lower virulence. The rough variant types were not observed to occur spontaneously at any phase of the natural infection.

DISCUSSION.

Throughout the studies of spontaneous Friedländer-like bacillus infection among special mouse populations, the conditions of experimentation were so controlled as to permit an analysis of the main factors determining the amount and spread of the disease. The cultures of Friedländer-like bacilli used in the present study were obtained from mice in these special populations, during various epidemic and endemic phases of the infection.

Hence, special attention is directed to the virulence titrations recorded in this paper, since they show that strains of bacilli derived from mice at epidemic and postepidemic periods, are of one type, of uniform and constant virulence, and in these respects conform to the observations made in earlier studies on mouse paratyphoid (1, *e*) and enteritidis (1, *a*) infections, and rabbit Pasteurella (1, *f*) infection.

The results of virulence determinations reported by us are not in

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demic group 8.11 ± .14; and interepidemic group 6.14 ± .12. Clearly there is a difference between the duration of life of the third and remaining groups. The bacteria of the interepidemic period appear to kill more rapidly and with less variation in time than those in the preepidemic or peak epidemic periods. The drawing of this conclusion does not appear to be justified, however, for if the data be examined, a clear association between the experimental groups is noted when these groups are arranged by dates. This association is largely due to the relatively long time which was taken to kill on the date of the first experiment and the short duration of life for the last two experiments. When the influence of the factor of time of experiment is removed the differences between the three groups of bacteria in the time with which they kill become insignificant, or there is no detectable influence of time of isolation of the bacteria and the duration of life for those mice which die."

accord with the views expressed by Topley, Lockhart, and Greenwood, in their papers on experimental epidemiology. These authors, it is true, have discussed the subject of virulence rather from the theoretical than from the experimental point of view, since they report few actual titrations of the mouse typhoid organisms with which they deal.

In order to make this divergence of attitude clear, we venture to review the papers of Topley and his associates at some length.

In 1919 (18), a series of tests of virulence, without control observations, is reported by Topley, in which mice of varied breeds, without known history affecting age, food, surroundings, and exposure to infection, were employed. Small numbers, all kept together in one cage, were used for each test. The culture employed was a stock strain of Pasteur Institute Danysz bacillus, fed on bits of bread. Survival of the animals following this procedure was, for the most part, random and unpredictable, and yet the varied effects were taken as indicating fluctuations in microbic virulence taking place during the experiments. No other possible factor seems to have been considered, and the results were cited as evidence in favor of the thesis stated in the earlier paragraphs of Topley's paper, that "an increase in the pathogenicity of the specific parasite is an essential factor in the rise of epidemics" (18).

That this view was premature and not conclusive is indicated in a later paper by Topley's associate, Lockhart (19), who states that "these experiments (Topley's) were not, however, especially designed to permit the observation of variations in bacterial virulence, other conditions being kept constant, and the conclusions based upon them must, therefore, be regarded as purely inferential and tentative" (page 50).

As, however, Lockhart did not control the host factor in his own tests, and remarks, with reference to his own test inoculations by way of the natural mouth portal of entry, "It is quite certain that, using any number of mice between 20 and 50, the form of the (mortality) curve will vary widely and in a random manner" (page 60), he resorted to intraperitoneal inoculations. This procedure, although useful in some ways, throws no light on the behavior of host and microbe under natural conditions. (Lange's recent experiments also demonstrate this (21).) Four of five of Lockhart's attempts to increase virulence by animal passage were reported as negative; nevertheless he states that while he has "not succeeded in controlling, or varying at will, the virulence of a given strain of *B. aertrycke*" (page 82), he does regard his tests as showing that "fluctuations in the virulence of *B. aertrycke* certainly occur" (page 81).

Another of Topley's collaborators, Wilson (20), later confirmed a view held by us in stating that there is no experimental evidence to support the hypothesis of a geometric rise and fall of bacterial virulence.

In other words, the point of view which has grown out of our own experiments (1, *a*, 1, *e*, 1, *f*, and the present paper) and the latest views of Topley's collaborators are coming more and more nearly to agree with each other.

Turning again to the results reported in the present paper, one may say that when the conditions of host and pathogenic organism are adequately controlled, it is found that mice react to the Friedländer-like bacilli in the same manner as has been stated to occur with other native animal infections,—that is, they do actually differ in respect to their native and acquired ability to resist infection (1, *g*). In the case of the Friedländer-like bacillus infection, as in the instances of other mouse and certain rabbit infectious diseases, we regard the several kinds of clinical manifestations—the septicemic, pulmonary, local or nasal infections, and the carrier state—not as sharply separated clinical conditions, but altogether as the sum total of the disease, each particular manifestation of which representing the momentary degree of resisting power of individual animals affected.

SUMMARY AND CONCLUSIONS.

A spontaneous respiratory disease of mice incited by Friedländer-like bacilli has been described. The bacilli inducing the disease, while morphologically and culturally indistinguishable from the usual varieties of Friedländer bacilli, are antigenically distinct from the common type strains known. The bacilli grow better in cultures at 23°C. than at 37°C.

The disease in mice displayed an incubation period of about 48 hours. About 50 per cent of an exposed population succumbed to septicemic and acute hemorrhagic, pneumonic processes. Among the exposed animals were some individuals which remained apparently well and carried the pathogenic bacilli in their nasal passages.

The several manifestations of the spontaneous disease were reproduced by instilling small numbers of the cultured bacilli into the nasal passages. 48 hours after inoculation, certain mice had already succumbed; the deaths continued to occur, so that by the end of the 2nd week, 70–80 per cent of the animals had died. Among the survivors certain carriers of the bacilli in the nasal passages occurred; a few appeared entirely refractory to the infection. The succumbing

mice showed at autopsy and by culture septicemia and diffuse pneumonic inflammation.

No fluctuation in virulence could be detected in bacilli derived from mice while the disease was spreading spontaneously at the periods of epidemic rise, interepidemic interval, or postepidemic quiescence. Moreover, the bacilli cultured from the nares of apparently healthy carriers were equally pathogenic with those taken from the blood and lungs of animals succumbing quickly.

No rough colony variants were cultured at any phase of the spontaneous disease, although they were readily obtainable by artificial culture. The variant strains proved stable and of low virulence.

This Friedländer bacilli infection in mice takes several clinical courses, depending on variations in host reaction and not depending on bacterial variation. The particular type of infection manifested is determined by the degree of resisting power displayed by the infected animals at the moment that the infection occurred and progressed.

We desire to thank Dr. L. Julianelle for his kindness in titrating the mouse Friedländer strains in his type sera, and Dr. John W. Gowen for analyzing mathematically the experimental data on which a part of this paper is based.

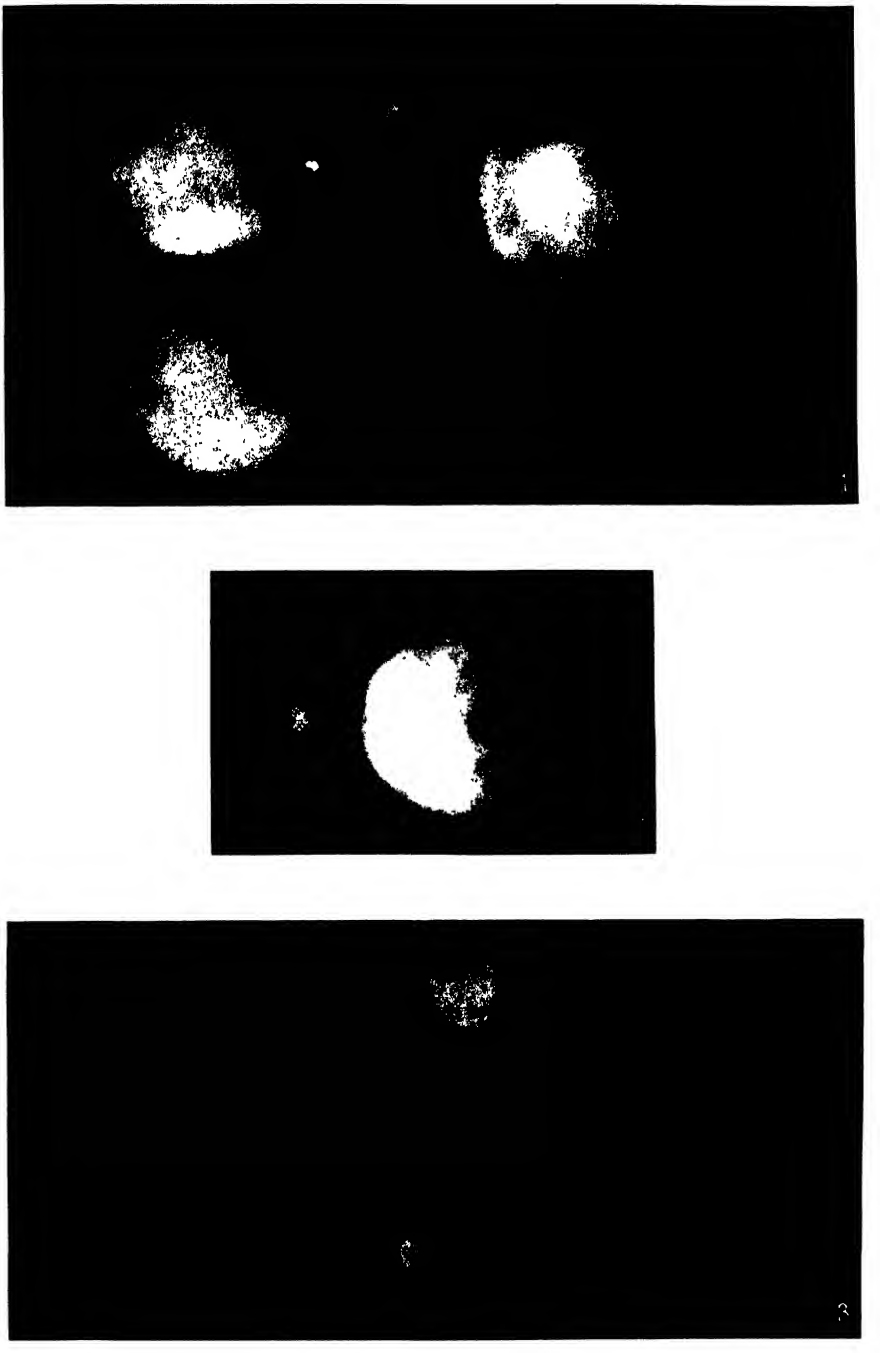
BIBLIOGRAPHY.

1. Webster, L. T., and associates: (*a*) *J. Exp. Med.*, 1927, xlvi, 847, 855, 871, 887; (*b*) *J. Clin. Inv.*, 1927, iii, 465; (*c*) Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 195; 1926, xliii, 161; (*d*) *J. Exp. Med.*, 1926, xliii, 555; (*e*) *J. Exp. Med.*, 1923, xxxvii, 781; xxxviii, 33, 45; (*f*) Webster, L. T., and Burn, C., *J. Exp. Med.*, 1926, xliv, 359; (*g*) *J. Exp. Med.*, 1923, xxxvii, 231; 1924, xxxix, 129, 879; 1926, xliii, 573.
2. Abel, R., and Hallwachs, W., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 515.
3. Pfeiffer, R., *Z. Hyg.*, 1889, vi, 145.
4. Klein, E., *Centr. Bakt.*, 1889, v, 625; 1891, x, 619, 841.
5. Weaver, G. H., *Tr. Chicago Path. Soc.*, 1897–99, iii, 228.
6. Skschivan, T., *Centr. Bakt., 1. Abt.*, 1900, xxviii, 289; *Centr. Bakt., 1. Abt., Orig.*, 1902–03, xxxiii, 260.
7. Perkins, R. G., *J. Exp. Med.*, 1900–01, v, 389.
8. Sachs, M., *Centr. Bakt., 1. Abt., Orig.*, 1902–03, xxxiii, 657.
9. Schilling, C., *Arb. k. Gsndhtsamte*, 1902, xviii, 108.

10. Toyama, C., *Centr. Bakt., 1. Abt., Orig.*, 1902–03, xxxiii, 273.
11. Aujeszky, A., *Centr. Bakt., 1. Abt., Orig.*, 1904, xxxvi, 603.
12. Xylander, *Arb. k. Gsndhtsamte*, 1906, xxiv, 196.
13. Holman, *J. Med. Research*, 1916, xxxv, 151.
14. Branch, A., *J. Infect. Dis.*, 1927, xl, 533.
15. Stillman, E. G., and Branch, A., *J. Exp. Med.*, 1925, xli, 623.
16. Julianelle, L. A., *J. Exp. Med.*, 1926, xliv, 113, 683, 735.
17. Hadley, P., *J. Infect. Dis.*, 1927, xl, 1.
18. Topley, W. W. C., *Lancet*, 1919, ii, 1, 45, 91.
19. Lockhart, L., *J. Hyg.*, 1926, xxv, 50.
20. Wilson, G. S., *J. Hyg.*, 1926, xxv, 142.
21. Lange, B., and Yoshioka, M., *Z. Hyg. u. Infectionskrankh.*, 1924, ci, 451. Lange, B., and Nowosselsky, W., *Z. Hyg. u. Infectionskrankh.*, 1925, civ, 648.

EXPLANATION OF PLATE 33.

FIGS. 1–3. Mouse strains of Friedländer's bacillus. Smooth and rough colonies. × 5.



Photographed by Louis Schmidt.

(Webster: Friedänder bacillus-like infection.)

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ON INDIVIDUAL DIFFERENCES IN HUMAN BLOOD.*

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The existence of individual differences in human blood was shown long ago by means of normal isoagglutinins (1). Soon thereafter variations were found also in the blood of animals (goats) through the use of immune sera prepared by injecting goats with blood of different individuals of the same species (2). These observations were later extended to other animals.

Curiously enough the reactions with normal human isoagglutinins do not occur in a, so to speak, haphazard manner but they separate the human bloods into four sharply defined groups designated as O, A, B, and AB. (1, 3).** It seems superfluous to describe the well known properties of these four groups.

In contradistinction to the simple scheme encountered with human blood, are the findings of Todd and White (5) who studied the serum of cattle immunized against cattle plague with the blood of infected animals. Taking advantage of the isohemolytic properties of such sera they discovered a remarkable variety in cattle blood; and similar conditions have been observed in the blood of chickens (Landsteiner and Miller (6)).

Attempts have been made also to discover further differences in human blood in addition to the group distinction. Evidence along this line has been furnished by von Dungern and Hirschfeld (7) in experiments with absorbed normal animal sera. It is difficult however to apply this method to a systematic study and the work was not carried on further. Following the technique of von Dungern and Hirschfeld we found some differences (see also Landsteiner and

* See the preliminary reports in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 600, 941.

** In the present paper the nomenclature adopted by the American Association of Immunologists is used (4).

Witt (8)) aside from the conspicuous ones characterizing the groups, or those ascribable to the subdivision of group A, or due to the agglutinable factor found by Schiff (9) in blood cells of group O (*cf.* Witebsky and Okabe (10), Hirszfeld (11)). Our experiments were not regularly successful and frequently variations of smaller order could not be confirmed on repetition of the tests.

Another way of showing distinctions among individual bloods but also somewhat difficult of application, was found in the reactions of "cold agglutinins" of normal human sera (12–14).

The present paper concerns itself with a method of differentiating human bloods which yielded clear-cut and reliable results. It is based upon the use of immune agglutinins.

Using this technique Hooker and Anderson (15) found that immune-sera produced in rabbits by injection of blood of group O still contained agglutinins for O blood after absorption with cells of any other group. The authors were inclined to explain this effect on the assumption of a property common to group O bloods.

EXPERIMENTAL.

Our first observations were made in experiments with stock anti-human blood immune sera from rabbits. Out of forty-one sera four were found that, after exhaustion with one sample of human blood, still contained agglutinins acting on a majority of bloods of all four groups while other bloods were not agglutinated. These tests showed the existence of an agglutinable property unrelated to the isoagglutinogens A and B, and differing from the latter in that there was not found a corresponding isoagglutinin in human serum. Naturally endeavors were made to produce immune sera endowed with the peculiar property described, by injecting rabbits with bloods possessing the new quality which may be designated as M. This was found to be rather difficult because only a few of the rabbits produce potent sera specific for M. However, on immunizing a sufficient number of animals, several such sera were obtained.

Some of the immune sera exhibited a different effect. When they were absorbed with blood of the type M+, the supernatant fluid reacted intensely and selectively on certain blood specimens, thus revealing a second agglutinable property (N).

The production of antibodies for N by injecting positively reacting bloods succeeded easily and some such sera were found among our supply of anti-human blood immune sera.

As was to be expected, anti-N agglutinins were not found in normal human sera. Also in normal animal sera we have not yet detected agglutinins for M or N.

An immune serum for a third agglutinable factor P was prepared by injecting blood (of colored individuals) selectively acted upon by absorbed normal rabbit and beef serum (according to the method of von Dungern and Hirschfeld).

In order to prepare specifically reacting agglutinin solutions, the inactivated immune sera in a dilution of 1:15 to 1:30 were treated with half the volume of packed, washed blood cells lacking the respective agglutinogens. A second treatment with the same or a smaller quantity of blood was required ordinarily to remove completely the agglutinins acting on human blood in general. The mixtures were allowed to stand for 1 hour at room temperature and were centrifuged. The fluids for N were prepared at first in this manner; subsequently, as will be explained below, the mixture of blood and serum was kept for ½ to 1 hour at 37–40°C.

The details of the procedure have to be determined in preliminary experiments and the absorbing blood must be selected with regard to the properties of the serum, *e.g.*, the presence of group agglutinins. Before setting up the main experiments the fluids were controlled by testing them with known bloods.

The tests were made by adding to 3 or more drops of the agglutinating fluids 1 drop of 2.5 per cent suspension of washed blood. The readings were made after the tests had stood for 2 hours at room temperature, or 1 hour at 37°C., if the fluids had been prepared at this temperature. The strength of the reaction is indicated as follows: F. tr. = faint trace; tr. = trace; ±, +, +±, etc. + signifies clumps visible without magnification or with a hand lens (magnification 6×) or clumps of medium size seen in the microscopic field (magnification 100×); ++ signifies large clumps seen with the naked eye and +++ complete agglutination.

For the production of immune sera freshly drawn and citrated blood (mostly of group O), after washing, was injected into rabbits at weekly intervals. The first injection of 3 cc. was given intravenously; the following injections of 4 cc. each, intraperitoneally. The sera were tested by absorption 6 days after the third and each subsequent injection. The animals were bled (mostly after four or five injections) the day following the tests when the sera had a sufficient content of the desired antibodies, i.e., when they gave powerful specific reactions after absorption. For the preparation of anti-M immune sera it seems preferable to inject bloods of the M+ N− type.

TABLE I.

The immune sera diluted 1:20 were absorbed with suitable blood lacking the particular agglutinogen, as described. The N agglutinins were prepared by absorption at room temperature (see page 769). Readings were made after 2 hours at room temperature.

Blood No.	815	816	817	819	821	822	823	824	825	826	828	829	830	831	832	833	834	835	836	837	838	839	840	841
Group	O	O	O	B	O	O	B	O	B	O	O	B	O	A	O	A	O	O	O	O	A	O	A	O
Immune serum 1; agglutinins for M	++±	+±	++±	++±	++±	0	++±	0	+++	++±	+++	0	0	+++	++±	+++	++±	+++	+++	+++	+++	++±	++±	0
Immune serum 18; agglutinins for N	0	tr.	0	+±	++	++±	0	+++	++±	±	++	++±	++±	+	+±	0	++	0	+±	++	0	++	+±	++±
Immune serum 5; agglutinins for P	+±	++	++	++±	+±	+±	±	+	++	+	+	+	0	++	+±	±	0	+	+	±	+	+	0	0

It was of considerable importance to have at one's disposal a number of individuals whose blood could be examined repeatedly. The work was facilitated also by keeping particular specimens in a mixture recommended by Rous and Turner (5 volumes of 5.4 per cent glucose solution, and 2 volumes of a 3.8 per cent sodium citrate solution, for 3 volumes of blood) (16). In this solution the erythrocytes were still agglutinable when the blood was kept sterile in the refrigerator for several weeks.

TABLE II.

Frequency of M in the Four Blood Groups.

Group		O		A		B		AB		Total Number	
Reactions for M.		+	−	+	−	+	−	+	−	+	−
Men	Number	299	64	285	48	114	21	34	15	732	148
	Percentage	82 4	17.6	85 6	14 4	84 4	15 6	69 4	30 6	83 2	16 8
Women	Number	115	40	80	17	32	10	13	3	240	70
	Percentage	74 2	25 8	82 5	17.5	76 2	23 8	81 2	18 8	77 4	22.6
Total Number		414	104	365	65	146	31	47	18	972	218
Percentage		79 9	20 1	84 9	15 1	82 5	17 5	72 3	27 7	81 7	18 3

TABLE III.

Frequency of N in the Four Blood Groups. Absorptions and Tests at 37°C.

Group	O		A		B		AB		Total No.	
Reactions for N	+	−	+	−	+	−	+	−	+	−
Total No.	162	48	143	46	28	10	6	3	339	107
Percentage									76 0	24.0

A representative experiment on twenty-four blood samples taken at random with exhausted immune sera containing agglutinins for M, N, and P, respectively, is given in Table I.

The frequency (in white individuals) of the positive and negative reactions for the property M and their occurrence among the blood groups are presented in Table II. The distinction between positive and negative bloods for M was regularly sharp when the exhaustion was made with suitably selected bloods.

It is seen from the table that positive reactions are much more frequent than negative ones. The percentage figures for the four groups do not deviate greatly from the total average except for group AB, but here the number of individuals examined is too low to warrant any conclusion. A similar remark may apply to the figures for the sexes.

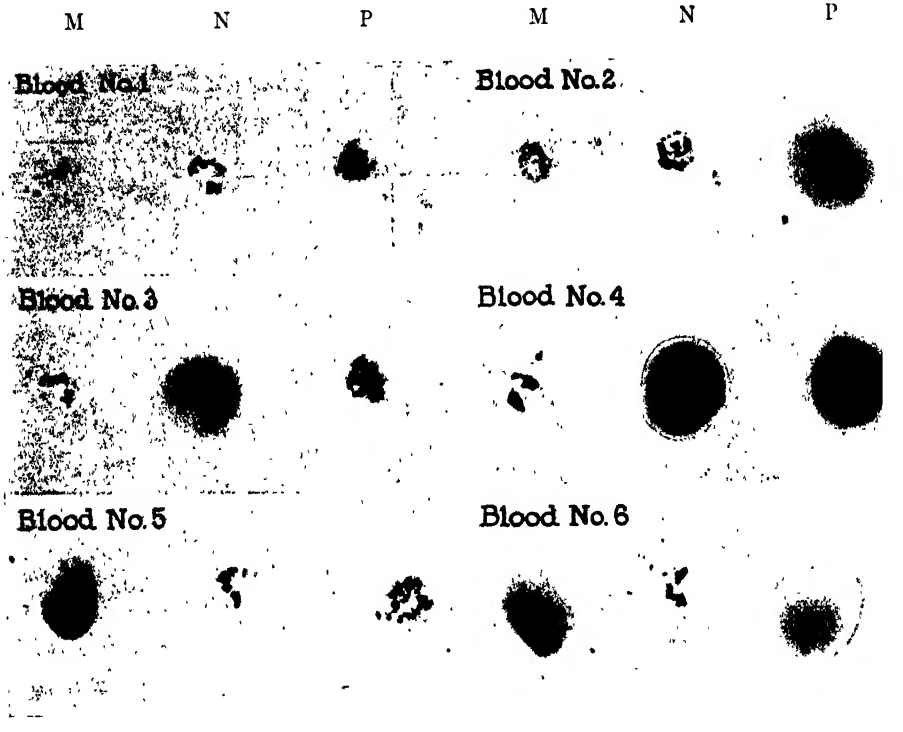

FIG. 1. Six selected bloods of group O tested with agglutinins for M, N, and P; natural size.

The distribution of the property N among the four blood groups is summarized in Table III.

For P our present results do not permit of a similar statistical survey but in general they indicate that there is no characteristic group distribution.

From the presence or absence of three agglutinogens, M, N, or P, there would follow eight possible combinations. Of these six actually

have been found in groups O and A. In groups B and AB some of the rarer types have not yet been found, most likely because of the comparatively smaller number of specimens completely examined.

The six combinations observed are illustrated in the photographs (Figs. 1 and 2). The tests reproduced were made with blood from

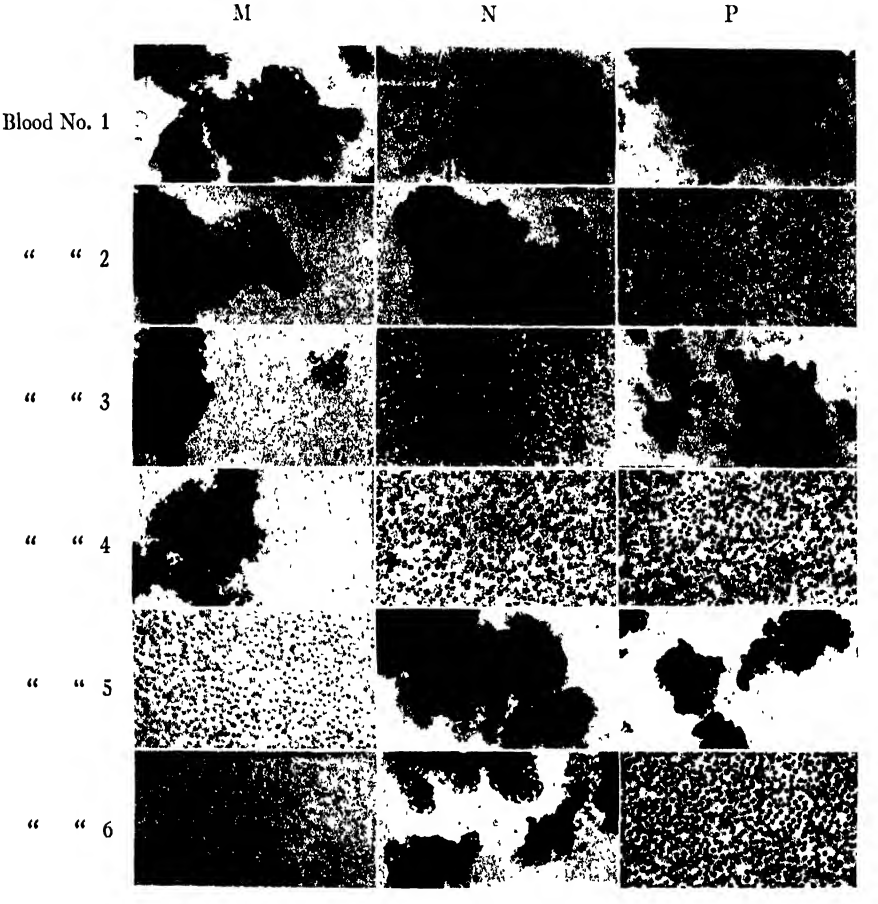

FIG. 2. The same tests as shown in Fig. 1, magnified 1:250.

six selected individuals all belonging to group O. The absorptions and tests for N were carried out at room temperature.

The two combinations not observed are those in which both properties M and N are absent. From the incidence of M− and N− bloods

TABLE IV.

Several immune sera absorbed with one M− blood (549). Sera 8, 21, and 22 diluted 1:15: Sera 1 and 20 diluted 1:20.

Agglutinins for M: serum No.	Group O								Group A										Group B		Group AB	
	201	205	235	244	252	262	266	279	199	220	242	500	549	566	568	570	591	592	23	560	272	522
1	+++	0	±	f. tr.	++±	+++	0	++	0	0	0	+++	0	0	++±	++	++±	++±	+	0	0	+++
8	++±	0	0	0	++±	++±	0	+±	0	0	0	++±	0	0	++±	++	+++	+±	+	0	0	++±
20	+++	0	0	0	+++	+++	0	++±	0	0	0	+++	0	0	+++	++±	++±	++±	+±	0	0	+++
21	+++	0	±	f. tr.	++±	+++	0	++±	tr.	0	f. tr.	+++	0	f. tr.	+++	++±	++±	++±	+	f. tr.	0	++±
22	++±	0	tr.	f. tr.	++±	++±	0	++	0	0	f. tr.	+++	0	0	+++	++	++±	+±	+	f. tr.	0	+++

TABLE V.

One Anti-M Serum Absorbed with Various M− Bloods.

Agglutinins for M prepared from serum 8 by absorption with various bloods			Group O						Group A								Group B				Group AB			
									A^2	A^1	A^2	A^2	A^1	A^1	A^1	A^2					A^1	A^1	A^2	A^2
Blood No.	Group	Subgroup	214	218	235	262	301	306	220	359	369	382	506	512	566	575	198	236	291	292	531	532	535	557
306	O		+++	+++	0	+++	0	0	f. tr.	+	±	++±	+++	+++	+	+++	0	0	0	++±	+	++±	+++	+++
359	A	A^1	+++	+++	0	++±	0	0	0	0	0	++	+++	+++	0	++±	0	0	0	++±	0	++±	++±	++±
566	A	A^1	+++	+++	0	+++	0	0	0	0	0	++	+++	+++	0	+++	0	0	0	++±	0	++±	++±	++±
220	A	A^2	+++	+++	0	+++	0	0	0	±	0	++	+++	+++	±	+++	0	0	0	++±	tr.	+++	+±	++
369	A	A^2	++±	++±	0	++±	0	0	0	f. tr.	0	++	++±	++±	f. tr.	++±	0	0	0	++	0	++	+±	++

one would expect an incidence of about 4 per cent bloods lacking both properties. This discrepancy evidences a negative correlation between the two agglutinable properties M and N, a conclusion supported by the fact that invariably M− bloods reacted intensely with the N reagent.

To form a correct judgment of the significance of the phenomena it was of importance to examine in how far they are influenced by variations in the reagents. In the first place a comparison was made of several anti-M immune sera in order to establish whether we were dealing with a definite agglutinable property or whether the results change contingent upon the special serum employed. The following experiments in which five anti-M immune sera were absorbed with the same blood specimen furnish proof for the first alternative (Table IV).

It is evident from the table that the strongly positive reacting bloods are the same regardless of the serum employed. In no case is the reaction of a blood intensely positive with one anti-M serum and negative with another. Identical results have since been obtained with several additional immune sera. Whether there is any disproportion in the strength of the reactions when M+ bloods are tested with a number of immune sera we are unable to decide as yet. With the sera 8 and 20 the reactions are almost uniformly strong or entirely negative. The remaining sera showed slight or weak reactions with some bloods which, doubtless, according to the other tests, lack the property M. These will be discussed presently.

In a second series of tests (Table V) one of the sera, No. 8, was absorbed with five different bloods, one of group O and four of group A, two of which belonged to subgroup AA^1 and two to AA^2*.

This experiment agrees with the former, for, no matter which blood was used for the preparation of the fluid, the strong reactions occurred with the same blood specimens. Hence it follows that all these reactions involve one sort of agglutinogen and its corresponding agglutinin.

The fluid prepared by absorption with blood of group 0 gives weak or moderate agglutination effects with all the bloods of group A or AB, evidently due to the presence in the immune serum of an agglutinin for A. These reactions are removed by absorption with blood AA^1 while after treatment of the immune serum with blood AA^2 there is still some agglutination with bloods AA^1 (or AA^1B).

* For the nomenclature of the subgroups see (8, 14).

Similar interfering reactions were encountered not infrequently throughout these studies. In general they were brought about by the presence in the sera of normal or immune antibodies for the group factors A and B, of antibodies acting by preference on bloods of group 0 (Schiff (9)) and by the coexistence in the same immune serum of more than one of the new agglutinins; besides there may be other agglutinogens not yet analyzed. Suitable absorption experiments serve to eliminate such additional antibodies.

TABLE VI, *a*.

Serum 20 diluted 1:15 was absorbed once for 2 hours at room temperature with half its volume of blood lacking the factor M. Some of the fluid obtained was removed and the remainder was divided into two equal parts. One of these two fractions was further absorbed three times at room temperature with half its volume of the M− blood, and the other was simultaneously treated in the same manner but with washed sheep blood; after each absorption a small amount of fluid was withdrawn for the tests. The last absorption was made at 0°C. and the fluid was obtained by centrifuging in a jacket containing ice and water.

The fluids of the first, fourth, and fifth absorptions were titrated in progressively doubled dilutions, using 3 drops of the liquid and 1 drop of 2.5 per cent suspension of M+ and M− blood. Readings were made after the tests were kept 2 hours at room temperature.

Fluid after	Absorbed with blood	Test with blood	Fluid diluted 1:				
			15	30	60	120	240
1st absorption	Human M−	M+	+++	++	+	f. tr.	0
		M−	0	0			
4th "	Human M−	M+	+±	+	f. tr.	0	0
		M−	0	0			
	Sheep	M+	++±	++	+	0	0
		M−	0	0			
5th "	Human M−	M+	+	f. tr.	0	0	0
		M−	0	0			
	Sheep	M+	++±	++	+	tr.	0
		M−	0	0			

The question whether the properties M+ and M− are of a qualitative nature was approached by means of repeated absorptions of the immune sera with M− blood. In the experiment recorded in Table VI, *a*, and Table VI, *b*, it was found that the antibody for M could

be gradually absorbed from serum 20, the greatest effect being obtained at low temperature while in serum 72, even after several absorptions, there was only an indifferent diminution of the specific agglutinin, not more marked with human than with sheep blood which was used as a control. Consequently the property M is characterized as a particular agglutinogen according to serological terminology.

Some anti-M sera were found whose specificity could be recognized by different degrees of agglutination when the exhausted sera were tested with the two sorts

TABLE VI, *b*.

An experiment similar to the preceding was made with anti-M immune serum 72. This serum diluted 1:30 required two absorptions with human M− blood for removing the common agglutinins. After withdrawing some of the fluid it was divided in two portions and the experiment was continued as above with human blood and sheep blood. The last absorption was carried out at 0°C. The fluids of the second, fourth, and fifth absorptions were tested as before.

Fluid after	Absorbed with blood	Tested with blood	Fluid diluted 1:				
			30	60	120	240	480
2nd absorption	Human M−	M+	+++	+++	+	+	0
		M−	0	0			
4th "	Human M−	M+	+++	++	+	+	0
		M−	0	0			
	Sheep	M+	+++	++±	+±	+	0
		M−	0	0			
5th "	Human M−	M+	++	+±	±	f. tr.	0
		M−	0	0			
	Sheep	M+	++	+±	±	0	0
		M−	0				

of corpuscles; they were not good for further work because the species agglutinins could not be removed without a simultaneous loss of the specific action.

The action of several (six) anti-N immune sera on a series of bloods and the effect of the exhaustion at room temperature of two immune sera with various bloods were studied in an analogous manner as

described for the property M. Since the strong reactions occurred always with the same bloods the experiments warrant the assumption that here, too, a definite serological factor comes into play. This factor may be subject to some variation as will be discussed presently.

A difficulty was encountered, owing to the fact that on treating anti-N immune sera several times with N- bloods, there was a rather rapid diminution of the agglutinins for N. As a consequence it was not easy to estimate the adequate degree of absorption, although fluids of marked specificity could be prepared repeatedly (see Table I).

TABLE VII.

Each of four anti-N immune sera diluted 1:20 were absorbed three times with one-half volume of pooled blood of four individuals of group A lacking N. One set of the absorptions was performed at room temperature and the other at 37° (water bath). In the latter case the fluids were separated by centrifuging for about 1 minute at high speed in a jacket of warm water (about 50°): At the end of the centrifuging the temperature of the water was 37° or but little below. The fluids were tested with six selected bloods of group A, two of which reacted negatively, two moderately, and two intensely.

Absorptions and tests at room temperature

Anti-N immune serum	Fluid after	Blood No,					
		806	1010	546	931	851	953
18	1st absorption	tr.	±	++±	++±	+++	+++
	2nd "	0	0	±	tr.	++	++
	3rd "	0	0	0	0	±	±
22	1st "	0	0	++	++±	+++	+++
	2nd "	0	0	±	±	++±	++
	3rd "	0	0	f. tr.	0	+	±
26	1st "	f. tr.	f. tr.	++	++±	+++	+++
	2nd "	0	0	++	+±	++	+++
	3rd "	0	0	+±	+	++	++
61	1st "	tr.	0	++±	+++	+++	+++
	2nd "	0	0	±	+	++±	++±
	3rd "	0	0	tr.	f.tr.	+	+

TABLE VII—*Concluded.*

Absorptions and tests at 37°

Anti-N immune serum	Fluid after	Blood No. 806	1010	546	931	851	953
18	1st absorption	++	+±	++±	+++	++±	++±
	2nd "	f. tr.	0	++±	++±	+++	+++
	3rd "	0	0	++	+±	++±	++±
22	1st "	±	+	++	+++	+++	+++
	2nd "	0	0	++	++	+++	++±
	3rd "	0	0	+±	+	++±	++±
26	1st "	++	+±	++±	++±	+++	++±
	2nd "	+±	+	++±	+++	+++	+++
	3rd "	0	0	++±	++±	+++	++±
61	1st "	++	++	+++	+++	+++	+++
	2nd "	+	±	++±	++±	+++	+++
	3rd "	tr.	f. tr.	+±	++	+++	+++

The technic was improved by carrying out the absorptions and also the tests at 37° or 40° C.* Under these conditions the N antibodies are diminished by repeated absorption with N- bloods at a slow rate (Table VII) and the results are generally satisfactory. On random selection of anti-N sera and absorbing bloods, also weak or moderate reactions are apt to occur with bloods that react negatively when other immune sera or absorbing bloods are chosen. This may be due to quantitative or qualitative variations in the agglutinogen N aside from other reactions as discussed above for the property M (p. 766).

The agglutinable property designated as P has not been studied extensively. Doubtless the reactions as presented in Table I are different from those for M and N and are independent of the group agglutinogens A and B; furthermore we found a characteristic distribution of P in white and colored individuals.** But it has not been

* With this method, incidentally, a considerable proportion of stored antihuman blood immune sera were found to contain smaller or larger fractions of N agglutinins.

** See *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 941.

established that parallel results can be obtained with several immune sera which would define a single quality.

A serum of an individual in group B containing an abnormal isoagglutinin was recently described by Ottenberg and Johnson (17). Having had the opportunity to examine this serum, we found in conformity with the authors named, agglutination reactions (of moderate to weak intensity) with numerous bloods of groups O and B. By absorbing this serum with certain bloods A, it was possible to show that the abnormal isoagglutinin acted also on the majority of group A bloods.

These isoagglutination reactions do not coincide with the reactions for M, N, or P.

The observations described open the possibility of making an individual diagnosis of human blood for forensic purposes in cases in which this could not be done hitherto. In preliminary experiments the properties M and N could be demonstrated in blood kept in a dry state (on glass) for several weeks. The method consisted in absorbing specifically reacting fluids with stromata prepared from small amounts (50 mg.) of the dried blood.

Immunization Experiments.—To determine whether the formation of the antibodies described, depends solely on the individuality of the animal used or also upon the antigen injected, the following experiment was carried out: 12 rabbits were injected with blood of the type M+ N− and 6 with blood of the type M− N+, all belonging to group O. Out of the 12 animals after four injections of M+ N− blood there were 1 strong, 1 weak anti-M immune sera, and 5 of rather moderate strength. Two of the latter became strongly active after one or two additional injections. In this series there were 2 or 3 which had a weak action for N. (After a further injection 2 other animals receiving M+ N− blood developed antibodies for N, 1 of moderate and 1 of weak activity.)

Of the 6 animals receiving M− N+ blood all developed immune sera specific for N; 4 reacted strongly and 2 moderately; none of these contained antibodies for M.

The experiment shows that potent antibodies were obtained when the homologous agglutinogen was injected but that antibodies of weak activity were also produced by animals which had not received the corresponding antigen. These observations are not exceptional

and similar cases were reported, *e.g.*, by Weil and Felix (18) and Furth (19) with bacilli of the typhoid group (*cf.* Halber and Hirszfeld (20)).

Tests with Blood of Anthropoid Apes.—The presence in the blood of apes of agglutinogens indistinguishable from the human group fac-

TABLE VIII.

*Tests for M in Blood of Primates.**

Anti-M immune serum absorbed with human blood	Chimpanzees										Ourang		Gibbons					Man	
	1	2	3	4	5	6	7	8	9	10	1	2	1	2	3	4	5	M−	M+
M−	+±	++	++±	+±	+±	+±	+	++	++	++	+±	+	0	0	0	0	0	0	++±
M+	0	0	0	0	0	0	0	0	0	0	+±	+	0	0	0	0	0	0	0

* The blood of the first 6 chimpanzees, Ourang 1, and Gibbon 1 were examined at the same time.

TABLE IX.

Tests for N in Blood of Primates. Absorptions and Tests Were Made at 37°C. Serum 18 Was Tested after 2 Absorptions (18a) and 3 absorptions (18b).

Absorbed with blood	Immune anti-N serum No.	Chimpanzees				Ourang	Man	
		3	4	5	10		N+	N−
N−	12	tr.	tr.	tr.	tr.	+±	++±	0
	18a	++±	++	++±	++±	++	+++	f. tr.
	18b	+	+	+±	+±	+±	+++	0
	22	+	+	0	+	+	++±	0
N+	12	0	0	0	0	+±	0	0
	18a	0	±	±	0	++	tr.	0
	18b	0	f. tr.	tr.	0	+±	0	0
	22	0	f. tr.	0	0	+	0	0

tors A and B has been shown previously (Landsteiner and Miller (21), *cf.* von Dungern and Hirschfeld (7)). Some experiments were made to establish whether also the new agglutinable properties are to be found in the blood of anthropoids. To account for the existence of agglutinins in the blood of apes that cannot be removed by human

cells,* absorptions had to be made with bloods both lacking and possessing the factor in question; a positive reaction was indicated when agglutination took place in the first but not in the second instance.

The tests for P were negative in the blood of chimpanzees and 1 ourang. As to the quality M, it appeared to exist in the erythrocytes of each of 10 chimpanzees, but not in the blood of 5 gibbons (1 *Hylobates lar*, 3 *Hylobates leuciscus*, 1 *Symphalangus syndactylus*) and 2 ourangs (Table VIII). The reactions for N in the blood of chimpanzees were distinctly positive with one of the immune sera, but moderate or faint with two other sera tested (Table IX).

That the properties M and N in the blood of chimpanzees and man are related though not entirely identical is seen from the results with the various anti-N immune sera and from the observation that one anti-M serum very active for human blood acted on chimpanzee blood positively but markedly less so than the other sera.

Of the lower mammalians and birds there were examined for the property M: 2 macacus rhesus, 2 vervets, 1 baboon, 1 sapajou, 1 lemur; 1 horse, 4 cattle, 1 sheep, 2 pigs, 1 dog, 1 cat, 25 rabbits, 2 guinea pigs, 2 rats, 1 mouse; 1 duck, 1 chicken, and 1 pigeon. For N only rabbits, 23 in number, were examined (absorptions at room temperature). The tests gave negative results.

DISCUSSION.

In the present studies a method is described which led to the detection of well defined individual differences in human blood in addition to those characterizing the blood groups. On repeated examination of the same individuals the properties were constant. The reactions observed indicate the existence of distinct agglutinable properties. This is substantiated by the fact that for M there are no transitions between positive and negative reactions, since fluids with a titre of 1:64 for M+ blood did not react on M− blood; also blood negative for M has practically no affinity to the antibody of certain anti-M immune sera as shown by absorption experiments at room temperature.

With the two agglutinable properties N and P, an appreciable ab-

* See the tests with ourang blood.

sorption effect is brought about at room temperature also by blood negative in the agglutination test. The phenomenon can possibly be explained on the assumption that the antibodies for the particular agglutinogen and those for human blood in general are not entirely segregated but are partly in some sort of combination. This view is supported by the fact, already mentioned, that certain anti-M sera behave similarly while others stand repeated absorptions. There are other cases which may call for an analogous explanation. It has been shown, f. i., that from some normal human sera of group O, corpuscles A or B absorb not only the homologous but also a part of the heterologous isoagglutinins (8). Similarly agglutinin α^1 of human sera O and B can be removed by bloods of group A which lack A^1, particularly when the absorptions are made at low temperature.

The ultimate significance of the factors determined by serological reactions is still a matter for discussion and it is not at all certain whether to each factor there corresponds a special compound that might be isolated chemically (14). But there is evidence from a study of families that the agglutinable factors M and N are constitutional properties that are inherited as Mendelian characters.* As to their antigenic nature it is true that the immunization depends largely upon the individual response of the animal but, even so, the experiments indicate that the antibodies can be formed as a result of specific antigenic action.

The division of human blood into only four well defined blood groups was not in harmony with the manifold individual variations that become evident from the experiences on transplantation of normal tissues and tumors. Thus there was some reason to presume that the serological differences of cells and the transplantation specificity are phenomena of a different nature. This gap seems to be bridged by some previous findings (7, 14) and the present studies.

The six types aforementioned, if present, as is likely, in each of the four blood groups and in the subgroups of groups A and AB, differentiate 36 varieties of human blood. This number does not include the variations in the strength of the reactions which may also be determined constitutionally and it is improbable that we succeeded

* See *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 941, and unpublished results.

in detecting all differences which can be demonstrated by means of antibodies derived from rabbits. Possibly other animals when injected with human blood would furnish sera with new specific qualities. As stated already, with a reagent of a different sort, namely an abnormal isoagglutinin in a particular group B serum (Ottenberg and Johnson (17)), reactions were obtained which did not run parallel with those shown by the rabbit immune sera. To be sure this reagent is not available for general use, but still it further doubles the number of human blood varieties that can be differentiated.

Summing up all the known observations on the subject one is led to the opinion that almost every individual human blood may have its characteristic serological features (see Todd and White (5)) as already conjectured by von Dungern and Hirschfeld, although at present there is no actual method which would permit of an individual diagnosis of human blood. Conceivably this end could be achieved by the use of immune isolysins.

The results of studies concerning the heredity of the agglutinable properties and their distribution in populations of different racial composition are reserved for subsequent communications.

The findings dealt with have thus far no direct bearing on the selection of donors in transfusions because of the absence of corresponding agglutinins in normal human sera for the new agglutinogens.

SUMMARY.

A clear-cut differentiation of human blood, aside from the blood groups, could be made by means of special agglutinating immune sera. The observations point to the existence of several agglutinable factors for which no agglutinins are demonstrable in normal human sera. In view of the latter circumstance the results reported do not imply any change in the scheme of the four blood groups.

The body of serological evidence leads to the inference of a high degree of biochemical differentiation among individuals.

Again we are indebted for material used in this study to Dr. C. Floyd Haviland, Superintendent, Drs. I. J. Furman and John R. Knapp, First Assistant Physicians, and Miss Frances W. Witte, Superintendent of Nurses, of the Manhattan State Hospital, New York City.

BIBLIOGRAPHY.

1. Landsteiner, K., *Centr. Bakt., I. Abt., Orig.*, 1900, xxvii, 361; *Wien. klin. Woch.*, 1901, xiv, 1132.
2. Ehrlich, P., and Morgenroth, J., *Berl. klin. Woch.*, 1900, 453.
3. Decastello, A., and Sturli, A., *Münch. med. Woch.*, 1902, xlix, 1090.
4. Editorial, *J. Am. Med. Assn.*, 1927, lxxxviii, 1421.
5. Todd, C., and White, R. G., *J. Hyg.*, 1910, x, 185.
6. Landsteiner, K., and Miller, C. P., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxii, 100.
7. von Dungern, E., and Hirschfeld, L., *Z. Immunitätsforsch., Orig.*, 1910, viii, 526.
8. Landsteiner, K., and Witt, D. H., *J. Immunol.*, 1926, xi, 221.
9. Schiff, F., *Klin. Woch.*, 1927, vi, 303.
10. Witebsky, E., and Okabe, K., *Klin. Woch.*, 1927, vi, 1095.
11. Hirszfeld, L., *Ergebn. Hyg., Bakt., Immunitätsforsch., u. exp. Therap.*, 1926, viii, 462.
12. Bialosuknia, W., and Hirszfeld, L., *Przegladu Epidemjologicznego*, 1921, i, 437.
13. Guthrie, C. G., and Pessel, J. F., *Bull. Johns Hopkins Hosp.*, 1924, xxxv, 81.
14. Landsteiner, K., and Levine, Philip, *J. Immunol.*, 1926, xii, 441.
15. Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419.
16. Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.
17. Ottenberg, R., and Johnson, A., *J. Immunol.*, 1926, xii, 35.
18. Weil, E., and Felix, A., *Z. Immunitätsforsch., Orig.*, 1920, xxix, 24.
19. Fürth, J., *Z. Immunitätsforsch., Orig.*, 1922, xxxv, 133.
20. Halber, W., and Hirszfeld, L., *Z. Immunitätsforsch.*, 1926, xlviii, 34.
21. Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 853.

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ON THE DEVELOPMENT OF ISOAGGLUTININS FOLLOWING TRANSFUSIONS.

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Observations on individual differences in human blood aside from the 4 blood groups,[1,2] support the idea that in transfusion the blood of the donor and recipient is, as a rule, not identical in biochemical constitution even though belonging to the same blood group.

In the literature instances are reported in which the first transfusion was uneventful while a repeated transfusion with the same compatible donor was followed by severe symptoms,[3, 4, 5, 6, 7, etc.] There are, however, only casual remarks on the occurrence of atypical isoantibodies under such conditions.

We examined recently the sera of 4 individuals of group 0 and 3 of group A.* In 5 of these individuals, who had been transfused several times with the blood of the same donor of their own group, no abnormal agglutinins were observed. The 6th individual, of group 0, was transfused with blood (500 cc.) of the same group and the transfusion was repeated, using the same donor, since in the routine test no incompatibility was detected. Actually no unto-

[1] Landsteiner, K., and Levine, Philip, *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 600, 941.

[2] Landsteiner, K., and Levine, Philip, *J. Exp. Med.*, 1928, xlvii, 757.

[3] Lindemann, E. E., *J. Am. Med. Assn.*, 1914, lxii, 993.

[4] Sydenstricker, V. P. W., Mason, V. R., and Rivers, T. M., *J. Am. Med. Assn.*, 1917, lxviii, 1677.

[5] Libman, E., and Ottenberg, R., *Trans. College Phys.*, 1917, xxxix, 266.

[6] Waugh, W. G., *Brit. Med. J.*, 1919, ii, 39.

[7] Bowcock, H. M., *Bull. Johns Hopkins Hospital*, 1921, xxxii, 83.

* The terminology of the 4 blood groups used in this publication is that recommended by the American Association of Immunologists and by a committee of the National Research Council.[8]

[8] Editorial, *J. Am. Med. Assn.*, 1927, lxxxviii, 1421.

ward manifestation followed. On careful observation with a more delicate technique, however, an abnormal isoagglutinin was found in the serum of the transfused individual (after the 1st and 2nd transfusion) that reacted on numerous bloods and stronger on several others than on that of the donor. The reactions took place with bloods of group 0 and, as could be shown after removal by absorption of the common isoagglutinins, also with bloods of the other groups. Some of the results are recorded in the table. The tests were made by adding in small test tubes 1 drop each of saline, serum, and 2.5% suspension of washed blood cells. After the mixture stood for 2 hours at

TABLE I.

Tests with Post-Transfusion Serum No. 191.

	Group 0								Group B			
No. of blood specimen	191	195	231	235	248	255	257	261	207	208	1122	1226
Reaction	0	+	0	0	+±	0	+	0	±	0	+	0
	Group A								Group AB			
No. of blood specimen			213	233	520	726	851	1179	614	621	861	1206
Reaction			+	+	0	+	0	0	+±	0	0	0

The blood of the donor was No. 195.

room temperature readings were made by withdrawing a drop on a glass slide for microscopic examination. The reactions became distinct after about 30 minutes.

At the end of 2 hours the strongest reactions were similar to common isoagglutination of moderate strength. The reactions persisted at 37° with but little diminution. By adding a sufficient amount of sensitive blood the agglutinin proved to be absorbable.

The incidence of the reactions was as follows: Group 0, 20+ reactions, 40− reactions; Group A, 16+, 12− ; Group B, 6+, 9− ; Group AB, 3+, 6−. With regard to the occurrence in all groups, the reactions recall those obtained with immune agglutinins[1, 2] and

the abnormal isoagglutinin recently described by Ottenberg and Johnson.[9] When tests were made with numerous individual bloods no parallelism was observed between the effects of the immune sera mentioned and the transfusion serum. On comparing the latter with the serum described by Ottenberg and Johnson corresponding reactions were found in a number of specimens but distinct discrepancies in others.

After observing the abnormal reactions reported, we repeated the test with the serum of the recipient drawn prior to the first transfusion, and the cells of the donor. The results were again negative. On making the tests with the same serum and other bloods a trace of agglutination was noticed with 2 bloods which gave the strongest reactions with the post-transfusion serum. Hence one may conclude that an agglutinin of very slight activity was present originally in the serum of the recipient and that the amount of this agglutinin was increased as a result of the transfusion.

In the serum of another transfused individual (group A)† we found agglutinins which acted weakly on all bloods 0 and AA_2 only faintly on some AA_1‡ thus behaving in a manner similar to the cold agglutinin α^2 described previously.[10] The reactions disappeared at 37°.

On account of the observations reported it will be of interest to make a systematic study of post-transfusion sera in order to establish how frequently atypical agglutinins may occur.

By means of the immune agglutinins referred to above we were able to apply the principle used by Ashby[11] for the demonstration of donor's blood in the circulation of the recipient, also for cases where donor and recipient belong to the same blood group. In this way the foreign blood could be found for as long as 7 weeks after the transfusion. Investigations after a longer interval have not yet been made.§

[9] Ottenberg, R., and Johnson, A., *J. Immunol.*, 1926, xii, 35. *Cf.* foot-note 2.

† For this specimen we are indebted to Dr. M. Lederer of the Brooklyn Jewish Hospital, who noticed some irregularity in grouping this blood. The pretransfusion specimen was not tested by us.

‡ For the terminology see [10].

[10] Landsteiner, K., and Levine, Philip, *J. Immunol.*, 1926, xii, 441.

[11] Ashby, W., *J. Exp. Med.*, 1919, xxix, 267.

§ Since the publication of this paper positive tests have been obtained up to 14 weeks.

In connection with the reaction of the pretransfusion serum it may be stated that, on examining at room temperature (about 20°) numerous sera, some were found which gave atypical isoagglutination reactions. (*Cf.* Unger,[12] Guthrie and Pessel,[13] Jones and Glynn,[14] Landsteiner and Witt.[15]) One of these sera, of group AB agglutinated at room temperature blood of group 0 and AA^2 and to a lesser degree some bloods of group B. The reactions with this serum disappeared at 37°. It is intended to describe these observations in detail.

[12] Unger, L. J., *J. Am. Med. Assn.*, 1921, lxxvi, 9.

[13] Guthrie, C. G., and Pessel, J. F. P., *Bull. Johns Hopkins Hosp.*, 1924, xxxv, 33, 81, 126.

[14] Jones, A. R., and Glynn, E. E., *J. Path. and Bact.*, 1926, xxix, 203.

[15] Landsteiner, K., and Witt, D. H., *J. Immunol.*, 1926, xi, 221.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

X. LITMUS CONSTITUENTS AS VITAL STAINS: THEIR PREPARATION AND RELATIVE USEFULNESS.

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(Received for publication, January 30, 1928.)

The recent use of litmus as a vital stain for mammals (1–3) has emphasized the need for a more precise knowledge of the nature of this dye. We have therefore undertaken the separation and investigation of the coloring matters comprised in commercial litmus with special reference to their use as vital stains.

Rous (1–3) has described in detail the character of the staining obtained in rats and mice after intraperitoneal injections of litmus solutions. The animals tolerate the dye, which colors them markedly, and they may remain stained for many weeks. Some of the tissues are colored diffusely, while the hue of others is traceable to an intracellular segregation of pigment. The observed differences in hue at different situations has great interest since they may be taken to indicate local differences in reaction.

The material used by Rous was prepared from Kahlbaum's cube litmus by extraction with hot water, treatment with acetic acid, and precipitation with alcohol, according to a method given by Sutton (4), similar to one originally described by Mohr (4–6). It contained therefore, according to the statements in the literature, at least three important dyes: erythrolitmin, azolitmin, and erythrolein.

As early as 1840, Kane (7–10) isolated from commercial litmus four constituents: azolitmin, erythrolitmin, erythrolein, and spaniolitmin. He considered the two first mentioned to be the essential colorimg matters of the indicator. From time to time other authors have reported the isolation of slightly different pigments, Wartha (11–13) finding what he termed indigotin, and Scheitz (12, 13) a blue coloring matter distinct from azolitmin. On the whole, however,

workers agree that azolitmin and erythrolitmin are the chief sensitive indicator substances present in commercial "litmus," that erythrolein, a relatively insensitive indicator pigment, is always present, while very rarely spaniolitmin is to be found as well, which last can be distinguished from azolitmin only with great difficulty.

Preparation of "Whole Litmus" from Crude Litmus Cubes.

Nearly all of the methods employed for the purification of litmus eliminate from the crude material merely its grosser impurities, notably gypsum. They yield a substance applicable to chemical needs but too complex for physiological experiments. The final product of the method of Mohr (5, 6), or of the procedures given in standard texts on analytical chemistry (4, 14), which we shall term "whole litmus," has been repeatedly analyzed by us and found to contain, as coloring matter, over 90 per cent of erythrolitmin, a little azolitmin, and some erythrolein. There is present in addition a very small amount of gypsum. It was "whole litmus" that Rous used to advantage as a vital indicator.

The "whole litmus" just referred to, while non-toxic when derived from some commercial samples, is highly toxic in other instances. In order to avoid the complication thus introduced, as well as to obtain more precise limiting conditions, we have deemed it best before passing to physiological studies, to isolate and test on animals the individual indicator substances. After trying several methods we have found it best to extract the pigments in the acid state, as follows.

Isolation of the Constituents of "Whole Litmus."

Kahlbaum's litmus cubes were ground to a fine powder, well extracted with hot water, and the insoluble matter removed by centrifugation. The extract was reduced to a convenient bulk by evaporation, and concentrated HCl was added drop by drop to decompose carbonates. When the solution had just turned red, and effervescence had ceased, the volume was measured and 4 cc. of concentrated HCl was added for each 100 cc. of extract. This was then placed on a water bath for 2 or 3 hours, that is to say until no further precipitation occurred. The whole was poured onto a filter paper and the precipitate washed free from acid with hot water and allowed to dry. The dried precipitate was transferred to an extraction thimble of a Soxhlet apparatus and extracted with ether until all soluble material had been removed. The ether was withdrawn

from the Soxhlet, and extraction continued with 95 per cent alcohol until the alcohol came away colorless.

In this way three pigments were isolated. The etherial solution, when evaporated, yielded an oily red liquid, erythrolein. The alcoholic solution deposited, on evaporation, a bright red powder, erythrolitmin. The brownish red, insoluble residue which remained in the extraction thimble was azolitmin.

The erythrolein needed no further treatment. The filtered alcoholic solution of erythrolitmin was removed to an evaporating dish and carefully dried, washed with ether to remove any remaining erythrolein, and again dried. The azolitmin residue was purified by washing, first with ether, then with alcohol, and dried. Since the pigments in acid state are insoluble in water, and moreover, when powdered and dried do not keep as well as in the alkaline form, which is water soluble, they were converted to the latter. For this purpose thick suspensions were made in water of the dried, powdered pigments in the acid state. Sodium hydroxide in normal solution was added until the indicator became bluish. The material was then evaporated to dryness, and the pigment powdered and kept sealed. For the purposes of vital staining 2 to 5 per cent solutions were made up fresh when desired in 0.9 per cent sodium chloride solution or in water.

"Whole litmus" was prepared, according to the method of Mohr (6, 14), and the product, dissolved in 0.9 per cent sodium chloride solution, was also employed as a vital stain in experiments to be described below.

Characteristics of the Isolated Pigments.

General Characters.—The physical characteristics, solubilities, and colors of the pigment substances generally found in litmus, have been described in detail by previous authors (7, 12, 15). Erythrolitmin and azolitmin are of especial interest to the physiologist for the range of color change of these, which is from red to blue, lies within the presumptive pH range of many living cells. Erythrolein changes hue much less markedly, merely from red, on the alkaline side, to red-orange on the acid, at about pH 6.0 to 5.9. It is therefore only

of interest in that it complicates the use of the whole dye, litmus. In acid solution both erythrolitmin and azolitmin are a clear crimson-red, in alkaline solution blue. In the latter both are dichromatic, being a dark red-violet in very concentrated solutions.

Color Intensity.—The color intensities of erythrolitmin and azolitmin as prepared above are different. A given amount of erythrolitmin will color a solution more deeply than will an equal amount of azolitmin. This has been apparent from comparisons, in a Duboscq colorimeter, of equivalent concentrations of the two dyes, in the alkaline and acid forms, respectively. Both dyes had more than three times the color intensity of "whole litmus."

Colorimeter Readings. Acid Form.—Since the dyes are insoluble in water in the acid form, 0.025 per cent solutions in 10 per cent acid alcohol were compared. All the solutions were reddish orange, of so similar a hue as to afford a good color match.

Five 0.025 per cent solutions of azolitmin were made from specimens of the dye, each prepared from a different batch of litmus cubes. Read in the colorimeter against one another a noticeable variation in color intensity was found, the strongest solution possessing 1.5 times the intensity of the weakest. Similar solutions of erythrolitmin from five specimens of the dye, each from a different source, when compared with one another showed as considerable variations in color strength. A mixture of equal parts of all the azolitmin solutions, compared with a similar mixture of all the erythrolitmin solutions, showed the color strength of the latter to be 1.39 times that of the former.

Alkaline Form.—To study the color intensity of the two dyes in the blue state, comparisons of 0.025 per cent solutions in N/1 NaOH were made. The matching of color intensity was easy, despite the fact that the azolitmin was a slaty blue, and the erythrolitmin more azure.

Five alkaline 0.025 per cent solutions of the five specimens of azolitmin were compared in the colorimeter and so too were similar solutions of the erythrolitmin specimens. Of the alkaline azolitmin solutions the strongest possessed 1.6 times the color intensity of the weakest. Among the erythrolitmin solutions a slightly more uni-

form color intensity was found, the strongest being 1.4 times as intense as the weakest. Repeated comparisons of a mixture of equal parts of all the alkaline azolitmin solutions with a similar mixture of all the alkaline erythrolitmin solutions showed the erythrolitmin to be 1.45 times as strong as the azolitmin in color.

The Effect of Differing Concentrations of the Dye upon the Color in Vitro.

Owing to their dichromatism the dyes, erythrolitmin, azolitmin, and "whole litmus," may show colors varying with the concentration, a potential cause of error when they are used as indicators. The magnitude possible to such an error was investigated by comparing the colors of solutions of each of these indicators at differing concentrations. Solutions, varying in strength from 0.005 per cent to 0.05 per cent, were made up in Sörensen's phosphate buffer solutions (16) (M/15 primary potassium phosphate KH_2PO_4 and M/15 secondary sodium phosphate Na_2HPO_4, $2H_2O$), having a pH range from 5.5 to 8.04. In the case of all these indicators the diluted solutions were pure blue at pH 8.0, but with a tenfold increase in concentration appeared red-violet. A shift of the red, toward the alkaline side, had occurred corresponding to a change of 0.2 to 0.3 pH. On the acid side of the range, however, there was no observed difference in the color of the solution with differing concentration.

The degree of color change with alterations in the pH could not be so readily determined in extremely concentrated solutions of the dye as in dilute solutions, but it may, of course, be greater. In the employment of the indicators for tissue study, when they are often found concentrated in intracellular granules, one must take into account possible errors referable to differences in concentration of the dye. For this reason one cannot expect to obtain precise knowledge concerning the pH by the use of the dye. But in connection with this point,—and to anticipate slightly results to be described later,—it may be stated that after injection of the litmus indicators into the mammalian body the differences in color of the intracellular granules are so great that differences in concentration can be but a minor factor in determining them.

Salt and Protein Errors.

The presence of salt or protein in a solution may so affect the color range of indicators that they become of little value for determining its hydrogen ion concentration. The salt and protein errors of litmus and azolitmin have been studied in the past and found to be great (17); but the possibility presents itself that these errors may not be shared by erythrolitmin, the constituent of litmus employed for purposes of vital staining in the studies to be reported in a companion paper. We therefore determined the magnitude of the errors of this dye. These errors were found to be large. A specimen protocol follows which illustrates the magnitude of the salt error in solutions containing differing concentrations of sodium chloride.

TABLE I.

Concentration of NaCl	Apparent pH color	Actual pH (electrometric)	Difference (salt error)
per cent			
.2	7.21	6.99	.22
.4	7.21	6.93	.28
.8	7.21	6.85	.36
1.6	7.16	6.70	.46
3.2	7.12	6.58	.59
4.8	7.08	6.41	.55

Salt Error of Erythrolitmin.—Sörensen's M/100 phosphate buffer solutions were prepared, having a pH range from 5.5 to 8.34. To 10 cc. of each in a series of tubes of equal bore 0.3 cc. of a 0.1 per cent solution of erythrolitmin was added. In a companion series of tubes 5 cc. of M/50 phosphate buffer of pH 7.0 and 0.3 cc. of 0.1 per cent erythrolitmin solution were placed. To each of this latter series of tubes sufficient quantities of sodium chloride in 5 cc. of water were added to yield 0.2 per cent, 0.4 per cent, 0.8 per cent, 1.6 per cent, 3.2 per cent, and 4.8 per cent sodium chloride solutions in the total volume of 10.3 cc. The concentration of buffer in these tubes became therefore like that of the control series, M/100, and the amount of erythrolitmin present in all was similar. The addition of sodium chloride brought about a pronounced change of color, the solutions appearing more blue with each increase in the salt content, as compared with the controls. Electrometric determinations of the hydrogen ion concentration of all the solutions were then made. As the table given below shows, the solutions containing salt appeared bluer, that is to say, more alkaline than their true pH would warrant.

From this experiment it is obvious that the "salt error" of erythrolitmin is large. It is of importance to note that the error is toward the alkaline side, that is to say, in the opposite direction from the error of the phthalein indicators.

Protein Error of Erythrolitmin.—Erythrolitmin shares also the disadvantage of litmus and of azolitmin in that its protein error is large. This, like the salt error, causes the indicator to appear more blue in a solution containing protein than it should from the actual hydrogen ion concentration. For the experiments we employed blood plasma and egg albumin solutions. A specimen protocol will be detailed.

Sufficient M/15 phosphate buffer, pH 6.47, was added to clear rabbit plasma to give the mixture M/50 concentration of this buffer. Air was bubbled through for 30 minutes to remove CO_2 and then for another half hour a mixture of equal parts of air and hydrogen. The addition of a small amount of this plasma, 1 to 9 cc. of M/15 buffer solution, pH 6.9, containing erythrolitmin in the red-violet stage, caused a change in the color to a pure blue.

To determine the protein error of erythrolitmin it was deemed best to make up plasma-indicator mixtures in such a way that their final color would be neither blue nor red but violet, as is erythrolitmin alone in buffer solutions of hydrogen ion concentration between pH 6.9 and 7.4. Within this narrow range a change in color can be noted with each difference of 0.2 pH. Test solutions were therefore made up of a total bulk of 10.3 cc., in each of which was 0.3 cc. of 0.1 per cent erythrolitmin solution and varying amounts of M/100 phosphate buffer, pH 5.59, and of a mixture in equal parts of buffered plasma, M/50, and of distilled water free from CO_2 (see Table II). These mixtures contained, respectively, 10 per cent, 20 per cent, 40 per cent, 50 per cent, and 60 per cent of plasma and the concentration of buffer in all was the same, a shade less than M/100.

For color comparison a series of M/100 phosphate buffer solutions, having a pH range from 5.04 to 8.34, was used and to 10 cc. of each, 0.3 cc. of the 0.1 per cent erythrolitmin solution was added. Upon adding erythrolitmin to plasma one encounters in addition to the protein error, another error due to the salt present with the protein. To determine the share of this additional source of error, "control solutions" were made up, each containing in the same concentration of phosphate buffer the amount of salt presumably added to the "test solutions" with the plasma. This was done by employing Ringer's solution minus its sodium carbonate content, together with the necessary amount of M/15 phosphate buffer solution to bring the buffer concentration of the total mixtures to M/100. In this way "control solutions" were obtained which were nearly like the "test solutions" except in the respect that the latter contained protein. In order to find "control solutions" which matched the violet "test solutions," it was necessary to make up several series. For as the amount of plasma in the

TABLE II.

Erythrolitmin Color as Influenced by Blood Plasma Constituents.

1 "Test solution"	2 "Control solution" containing the plasma equivalent in salts	3 Color comparison with erythrolitmin in phosphate buffer	4 Error due to plasma (salt and protein error). pH in Column 3 minus pH in Column 1	5 Error due to the salt equivalent of the plasma. pH in Column 3 minus pH in Column 2	6 Error due to protein alone. pH in Column 4 minus pH in Column 5
1.0 cc. plasma, M/50 phosphate buffer content	1.0 cc. Ringer's solution				
1.0 cc. water	7.5 cc. water				
8.0 cc. M/100 phosphate buffer, pH 5.59	1.5 cc. M/15 phosphate buffer, pH 6.9	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 7.1	pH 0.76	pH 0.23	pH 0.53
0.3 cc. 0.1 per cent erythrolitmin solution	0.3 cc. 0.1 per cent erythrolitmin solution				
10.3 cc. buffer concentration M/100	10.3 cc. buffer concentration M/100				
pH electrometrically 6.42	pH electrometrically 6.95	pH electrometrically 7.18			
2.0 cc. plasma, M/50 phosphate buffer content	2.0 cc. Ringer's solution				
2.0 cc. water	6.5 cc. water				
6.0 cc. M/100 phosphate buffer, pH 5.59	1.5 cc. M/15 phosphate buffer, pH 7.4	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 7.7	pH 0.92	pH 0.23	pH 0.69
0.3 cc. 0.1 per cent erythrolitmin solution	0.3 cc. 0.1 per cent erythrolitmin solution				
10.3 cc. buffer concentration M/100	10.3 cc. buffer concentration M/100				
pH electrometrically 6.67	pH electrometrically 7.36	pH electrometrically 7.59			

3.0 cc. plasma, M/50 phosphate buffer content 3.0 cc. water 4.0 cc. M/100 phosphate buffer, pH 5.59 0.3 cc. 0.1 per cent erythrolitmin solution 10.3 cc. buffer concentration M/100 pH electrometrically 6.85	3.0 cc. Ringer's solution 5.5 cc. water 1.5 cc. M/15 phosphate buffer, pH 7.8 0.3 cc. 0.1 per cent erythrolitmin solution 10.3 cc. buffer concentration M/100 pH electrometrically 7.68	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 8.04 pH electrometrically 8.01	pH 1.16	pH 0.33	pH 0.83

"test solutions" was increased a greater and greater color shift toward the blue side occurred (see Table II).

The actual hydrogen ion concentration of all the solutions was determined electrometrically.

It was evident from such findings as are given in the table that the protein error of erythrolitmin is very large. When mixtures of the indicator are made with plasma or egg albumin, containing salt as well, the apparent error is still greater, for both salt and protein cause a change in the color of the indicator in the same direction, that is to say toward the alkaline side. As a result of this combined error a protein solution which is at about pH 7.0, that is to say, neutral, may appear frankly alkaline upon the addition of erythrolitmin, that is to say, blue.

When to 10 cc. of rabbit's plasma rendered CO_2 free and buffered as described above with sufficient M/15 phosphate buffer to give the mixture M/50 buffer concentration, 0.3 cc. of 0.1 per cent erythrolitmin solution was added, the color of the mixture matched that of erythrolitmin alone in phosphate buffer M/100 of pH 8.34. The actual pH of the protein mixture determined electrometrically was pH 7.20 and that of the buffer solution 8.19. The colorimetric reading was therefore in error by pH 1.0. In M/100 buffer solutions the color of erythrolitmin at pH 7.2 is red-violet, at 8.34 pure blue.

It has been noted elsewhere (1, 2, 3) that litmus segregated by living cells out of body fluids dyed blue with it is held in the red form. This is scarcely what one would expect were the intracellular reaction neutral or slightly alkaline for the influence both of protein and of salt would tend to render the litmus blue under such conditions. It is a matter of interest therefore to know the actual hydrogen ion concentration of protein mixtures rendered just sufficiently acid to bring the indicator to the red color it assumes in intracellular granules.

The protocol of one experiment on the point out of a number will be given.

To 3 cc. of buffered plasma, 1 cc. M/3 KH_2PO_4 solution (pH about 4.5) was added and 0.3 cc. of 0.1 per cent erythrolitmin solution. The resulting color was violet, not red. To the mixture phosphoric acid M/15 was added in just sufficient quantity (0.6 cc.) to bring about a brick red color like that of the erythrolitmin-stained granules frequently seen within tissue cells. The color of this

mixture matched that of erythrolitmin in M/15 phosphate buffer of pH 5.91. Electrometrically the pH of the plasma solution was found to be 4.81.

In connection with such findings it is to be recalled that the concentration error of erythrolitmin is in the opposite direction to that of the salt and protein errors, that is to say, the more concentrated the dye the more does it tend to appear red. It follows that an intracellular granule containing the dye in concentrated form need not have as great an acidity as that prevailing in the experiment above in order that it shall appear red.

From the foregoing results taken together it is obvious that in vital staining with erythrolitmin, one must content oneself ordinarily with the determination of relative differences in pH.

The Donnan Equilibrium Has No Effect upon the Distribution of the Litmus Derivatives.

A dye taken up by tissue cells becomes subject to the forces incident to the presence of protein substances. Our finding, that some changes in the color of the dyes result from changes in their concentration, made it important to study the influence of hydrogen ion concentration on the distribution of dye between fluid and protein, in mixtures of the two. A series of solutions were prepared containing powdered isoelectric gelatin and varying amounts of acid and alkali. "Whole litmus" or erythrolitmin was added to each. 20 hours later the fluid was separated by filtration and the amount of dye in it measured colorimetrically. All the solutions were found to contain the same proportions of the indicator. The conditions set up by the Donnan equilibrium did not change the relative distribution of the dye at the various hydrogen ion concentrations. From this finding it would seem that the probability of an error, caused by concentration changes due to the presence of protein, is slight.

A series of acid and alkaline mixtures were made up in 0.9 per cent NaCl solution, using N/1 HCl and N/1 NaOH in proper quantities to give N/5, N/20, N/40, N/80, N/160 to N/1280 acid in saline, and N/80, N/160, N/320, N/640, and N/1280 alkaline in saline. To 50 cc. of each of these 2 gm. of powdered isoelectric gelatin was added and 1 cc. of a 1 per cent solution of "whole litmus," just sufficiently alkaline to be in the blue state. The mixtures were each shaken 1 minute, packed in ice, and allowed to stand with the gelatin completely submerged in the fluid

for 20 hours. The litmus solution gave a red color to the gelatin and a blue to the supernatant liquid of the N/640 and N/1280 acid solutions immediately upon its addition to them; but after standing the color of the gelatin and supernatant liquid was uniform. The N/160 and N/320 NaOH solutions seemed to be close to the neutral point for the indicator, whereas the N/80 NaOH solution was alkaline to it, as shown by the distinctly blue color, and the N/640 NaOH solution acid, appearing red.

The supernatant liquids were separated from the gelatin by rapid suction filtration and the amount of dye present in each was estimated by adding to each 0.6 cc. of 40 per cent NaOH to convert the color to a strong blue to be read in a Duboscq colorimeter against a standard. For the latter purpose there was employed a solution containing 1 cc. of 1 per cent "whole litmus" to 50 cc. of saline with 0.6 cc. of 40 per cent NaOH. It will be seen that the amount of indicator equalled the total added to each of the gelatin mixtures.

The distribution of litmus was as follows:

Acid.

	N/80	N/160	N/320	N/640	N/1280
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
In supernatant liquid	54	53	52	47	49

Alkaline.

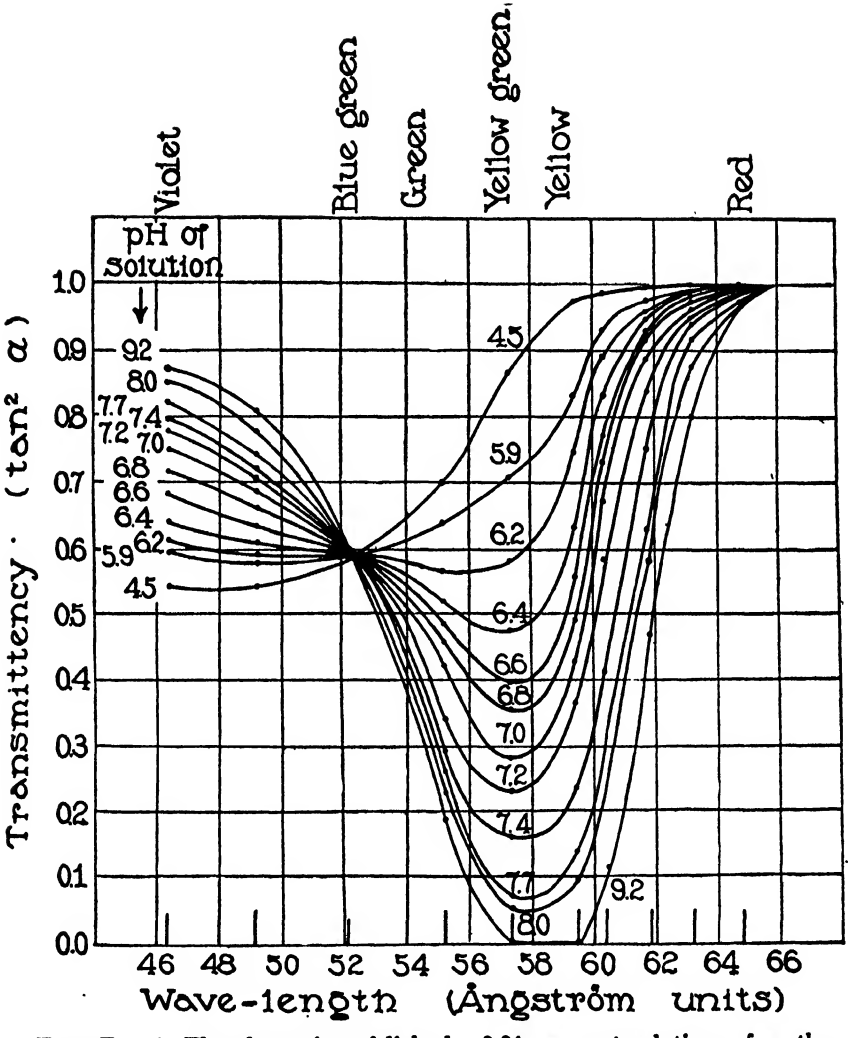
	N/1280	N/640	N/320	N/160	N/80
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
In supernatant liquid	49	56	52	61	58

The concentration of chloride ion in the supernatant liquids was found to be about the same in all. There was proportionately a little more in the gelatin. The determinations were made by adding an excess of silver nitrate to the solutions and titrating the amount of the excess with potassium thiocyanate, with iron alum as the indicator.

The behavior of erythrolitmin, studied in the same way, was found to be completely analogous to that of "whole litmus."

Indicator Properties of Erythrolitmin as Shown by the Spectrophotometer.

The property of certain dyes which enables them to serve as indicators is their ability to absorb different proportions of light in a given part of the spectrum at different hydrogen ion concentrations. The



TEXT-FIG. 1. The absorption of light by 0.01 per cent solutions of erythrolitmin, in buffers, at various hydrogen ion concentrations. The amount of light transmitted by the solutions is plotted along the ordinate, and along the abscissa, in Ångström units, light wave-lengths. Each curve records the variations in light absorption, in various regions of the spectrum, by a single solution of erythrolitmin at a definite pH. The regions of the spectrum at which readings were made are designated on the chart. Within the blue region of the spectrum, the more alkaline the solution, the more blue is transmitted. On the red side of the spectrum, the more acid the solution, the more red comes through.

phenomenon can be readily measured with the spectrophotometer when the instrument has been set to permit the passage of light of known wave-lengths, while allowing light of but one wave-length to pass at any given time. Studies of various dyes have been made in this way, and Brode (18) has recently applied the method to synthetic indicator substances, phenol red, brom cresol blue, and others.

A study of erythrolitmin and azolitmin with the spectrophotometer is of value in that it shows graphically the manner in which the substances act as indicators. It has proved furthermore that they are pure indicators, on a par in this respect with the synthetic dyes examined by Brode.

Four series of Sörensen's phosphate buffer solutions (16) were prepared, each containing 0.02 cc. of 5 per cent azolitmin or erythrolitmin to every 10 cc. As a control solution uncolored buffer was employed. Both were placed in glass cells 10 cm. long. The amount of light absorbed at certain arbitrary points on the spectrum was measured for each solution and a series of curves prepared. Those for erythrolitmin are shown in Text-fig. 1.

The instrument used was of the type first designated by König (19), modified by Martens (20), and made by Franz Schmidt and Haensch (Berlin). The apparatus is briefly described by Sheppard (21). A more detailed description can be found in the paper by Martens and Grünbaum (22), and by Grünbaum (23).

Briefly, the instrument provides for the passage of 2 equal beams of light from a single source, which pass through the solutions to be studied, then through 2 adjustable slits and a collimating lens and are finally refracted by a single flint prism. The emerging beams pass into the movable arm of the instrument which, by a graduated screw, can be placed at any angle thus admitting the light from any desired portion of the spectrum. Here the 2 refracted beams are polarized by a Wollaston calcite prism, passed through a biprism and brought to a focus by a telescope lens.

At the eyepiece 2 images, 1 from each slit, are seen polarized at right angles to each other. By rotating the crossed Nicol prism placed in the eyepiece the intensity of the light in each half of the field is reciprocally altered. Before making any observations the instrument is so adjusted that a movement of the Nicol prism to 45° from the zero point illuminates the 2 fields equally. This was also found to be the case, as it should be, at a rotation of 135°, 225°, and 315°.

Calculation: $\frac{S}{C} = \frac{\mathrm{Sin}^2 a}{\mathrm{Cos}^2 a}$

$\frac{S}{C} = \tan^2 a$

Where S = light transmitted through solution containing the dye.

C = light through the control solution.

a = angle of deviation (from 45°).

The amount of light transmitted through the colored solution will vary from 0, when all light is absorbed, to 1, when none is absorbed, that is to say, when the same amount of light comes through on the 2 sides. The light used was a Nernst filament set in series with a resistance coil. The readings of angular deviation were converted to Ångström units, by calibrating the instrument with known spectral lines and interpolating between.

The accompanying figure represents the findings for erythrolitmin. Azolitmin gave practically the same curves. The amount of light transmitted by the solutions is plotted along the ordinate and is simply the reading of $tan^2 a$, subtracted from 1, since $tan^2 a$ represents percentage of absorption. Along the abscissa are plotted the wave-lengths which correspond to the different parts of the spectrum in which the readings are made. Each curve records the variations in absorption of a solution of given pH and it is seen that they fall into a regular order with relation to each other.

It is of great significance that the curves of light absorption in solutions of different pH all cross at a single point, for this is characteristic of pure indicator dyes (18). In this case the point lies at a wave-length of 5250 Ångström units, which corresponds to a blue-green. Regardless of pH the proportion of light transmitted at this point is the same, 58 per cent. On the blue side of the spectrum, the more alkaline the solution, the more blue comes through. On the red side of the spectrum, the more acid the solution, the more red comes through.

The Relative Worth of the Litmus Dyes as Vital Stains.

Rous has recently used litmus as a vital stain in rats and mice (1, 2, 3). For weeks after intraperitoneal injection the tissues of these animals are still colored with it and it has retained its indicator properties. There are numerous differences to be seen which are presumably indicative of local differences in the reaction. We have made an effort in the course of the present work to throw more light on some of the phenomena observed after injection of litmus by comparing with them the effects of injection of the constituent coloring matters.

The litmus used by Rous (1) contained at least the three coloring matters, erythrolitmin, azolitmin, and erythrolein. *A priori* it would seem that the

heterogeneity of litmus might be disregarded when it is employed as a vital stain because of the circumstance recognized by others before us (15) that one of the constituents, erythrolitmin, makes up 90 per cent or more of the whole dye. But many authors have considered azolitmin the active principle of litmus, and conceivably this may have been true of the preparations with which they worked. Our material (Kahlbaum's litmus) which was the same that Rous employed yielded over 90 per cent of erythrolitmin, as already stated. Both azolitmin and erythrolitmin are indicators *in vitro* with practically the same virage and absorption bands, as we have pointed out, but the latter is a more intense dye and, as will be shown below, is more suitable for experiments *in vivo*. The third dye constituent of litmus, erythrolein, is a poor indicator for tissue study since the color change is merely from red to red-orange.

Procedure.

"Whole litmus," erythrolitmin, azolitmin, and erythrolein were separated out of litmus purified as already described, and injected into the peritoneal cavity of rats and mice. The individual dyes like the "whole litmus" were made up in 2 and 5 per cent solutions in 0.9 per cent sodium chloride solution. Sterilization was effected by heating test-tubes partly filled with the solutions in boiling water for 30 to 60 minutes.

The dyes were given with aseptic precautions. Graded doses were administered to many series of animals and special note was made of the rapidity and depth of the tissue staining which ensued. Special cages were employed for the collection of total urine. At various intervals, from half an hour to 10 days after the injections, the animals were put lightly under ether and guillotined. Autopsy was done at once, and the tissues examined. Fresh sections of the kidney, and frequently of other organs, made with the Valentine knife, were studied, and frozen sections were examined later. The kidney findings will form the subject of a succeeding communication. Staining of the tissues was best accomplished by injecting the dyes twice at an interval of 48 hours, killing the animal 2 days after the last injection.

Experimental Findings.

Erythrolein.—No generalized staining followed the administration of this dye. Nearly all of it was soon secreted into the urine which assumed in consequence an intense red color that failed to change notably on the addition of alkali. Moreover, erythrolein proved strongly toxic, causing death in doses of ½ mg. per gm. of body weight.

Azolitmin.—The 5 per cent solution of azolitmin in water or physiological saline proved to be thick and viscid. The indicator was absorbed slowly from the abdominal cavity and much of it could be

found there after 24 hours or more. As a consequence animals were not well colored after the injection of ¼ mg. of the dye per gm. of body weight. To obtain satisfactory staining as much as ½ mg. per gm. of animal was necessary, an amount which proved toxic and usually lethal.

Even the largest non-toxic dose stained the kidney but poorly. On the day following a single injection the glomeruli appeared intensely blue from the presence of the dye in the capillary tufts, and blue streaks in the kidney cortex represented the blood vessels in the midst of the pale, unstained parenchyma. The general staining of other organs was slight, but became definite after a second administration of the dye. When examination was made 2 days thereafter, the kidney contained much of the indicator as compared with the amount elsewhere. It was present in the tubule cells for the most part as large red granules though some cells contained blue ones.

Erythrolitmin.—Erythrolitmin has proved far superior to azolitmin or "whole litmus" as a vital stain. *In vitro* it exhibits a greater color intensity than azolitmin, as already demonstrated, and when injected into animals it is much less toxic, doses of ½ mg. per gm. of body weight being well borne. A generalized blue staining with the dissolved dye is caused by but ¼ mg. of erythrolitmin per gm. of animal. The injection of this amount causes a pronounced transient bluing of the body. The dye is employed to advantage in 2 per cent solution.

Within an hour after the injection of erythrolitmin into rats and mice blue areas appeared on the pads of the feet and in the ears of the injected animals, and within 2 to 3 hours the entire body was colored blue. After 24 hours little of the indicator could be recovered from the peritoneal cavity, practically all having been absorbed. At this time the animals were diffusely colored and the dye could be found in almost every tissue. Later much of it became segregated in granular form within cells. It is unnecessary to go into the findings in detail since they are identical with those recorded by Rous (1, 2, 3) for rats and mice injected with "whole litmus." After repeated doses the staining became intense and at autopsy most of the tissues appeared highly colored, the kidneys in particular containing large amounts of the dye. Within the tubule cells in

certain regions were granules stained a brilliant red, while in others they were blue, as observed by Rous (1).

The urine voided shortly after the injection was usually red, but, unlike the red urine found after injections with erythrolein, turned blue upon the addition of alkali. But soon excretion of the dye ceased, the urine becoming colorless and remaining so.

"Whole Litmus."—In the course of experiments, to be described in a later paper, numerous rats and mice were injected with "whole litmus" and examined after various intervals of time. The material proved toxic as compared with erythrolitmin and the staining of the tissues effected by it was less intense. The toxic effects would seem from our findings to have been due in considerable part to substances other than the indicator dyes. The various batches of "whole litmus," prepared by the same method, differed greatly in toxicity. Doses of ½ mg. per gm. of body weight of animal were often tolerated well, but at other times proved fatal. The specimens varied much in color intensity as well. Some contained much erythrolein, others little, and differences in the content of azolitmin and erythrolitmin were also found. The first urine collected after injection with "whole litmus" was red, often intensely so, but the addition of alkali caused no change to blue. From the characters of erythrolein, above described, one may conclude that the red color of the urine was due to the excretion of the erythrolein portion of the "whole litmus." Some part of the relative toxicity of the latter may also be ascribed to its content of this pigment.

DISCUSSION.

Caution must be exercised in the interpretation of findings with erythrolitmin and azolitmin when used as vital stains, despite the fact that the light absorption phenomena of these dyes, when prepared as described above, place them on a par as concerns purity with synthetic indicators such as phenol red (18). Both of the dyes are colloidal and they tend to become segregated within the cells in granules. They are dichromatic as well, and because of this may give erroneous information when variously concentrated within cells. The total error from this source and from the presence of salt and protein is smaller, however, than one might expect. And our experi-

ments on the distribution of erythrolitmin and "whole litmus" between a protein and the fluid bathing it, as exemplified by gelatin and water, fail to demonstrate any important influence of the Donnan equilibrium.

While no accurate inferences concerning the absolute pH within living tissues can be drawn through the use of these dyes, changes in the relative reaction can be followed with ease. It is with this in mind that we have studied the indicators.

It is evident from our experiments that one may ascribe the tissue staining described by Rous (1, 2) almost wholly to the erythrolitmin content of the "whole litmus" that he employed.

SUMMARY.

We have devised methods for the separation and isolation of the important indicator constituents of litmus, azolitmin, and erythrolitmin, with a view to employing them as vital stains. Analysis of the color intensities of these dyes shows slight differences in them, azolitmin being the weaker pigment, weight for weight. Study of a third coloring matter, erythrolein, which exists in litmus has shown it to be an unsatisfactory indicator, and toxic for animals.

Analyses with the spectrophotometer of the absorption of light by erythrolitmin and azolitmin, prepared by our methods, and tested over a wide acid-alkali range, show them to be pure substances, comparable in this respect with synthetic indicators.

The errors in the interpretation of the indicator phenomena on vital staining, which are incident to changes in the concentration of the dyes, are so slight as to be negligible. The salt and protein errors on the other hand are large. The factors responsible for the Donnan equilibrium fail to influence the distribution of the indicators between fluid and gelatin.

Erythrolein was found useless when employed for vital staining, and azolitmin proved unsatisfactory since it colors poorly and is toxic. But erythrolitmin can be used to great advantage. It is readily absorbed, and in non-toxic doses stains intensely. The range of pH at which it changes from red to blue fits it for the demonstration of changes in the reaction of living tissues. By reason, however, of the

salt and protein errors to which it is liable, the pH cannot be accurately ascertained.

Intravital staining with erythrolitmin yields results similar to those following injection of purified "whole litmus."

BIBLIOGRAPHY.

1. Rous, P., *J. Exp. Med.*, 1925, xli, 379.
2. Rous, P., *J. Exp. Med.*, 1925, xli, 399.
3. Rous, P., *J. Exp. Med.*, 1926, xliv, 815.
4. Sutton, F., Systematic handbook of volumetric analysis, Philadelphia, 10th edition, 1911.
5. Mohr, F., Lehrbuch der chemische-analytischen Titriermethoden, 7th edition, 1896, cited by Classen (6).
6. Classen, A., Theorie und Praxis der Massanalyse, Akademische Verlagsgesellschaft m. b. H., Leipsic, 1912, 32.
7. Kane, *Ann. Chem. u. Pharm.*, 1840, xxxvi, 324.
8. Kane, *Roy. Soc. Tr.*, 1840, 298.
9. Kane, *Pharm. Centr.*, 1841, xii, 561.
10. Kane, *Ann. chim. et phys.*, 1841, ii, series 3, 129.
11. Wartha, V., *Ber. chem. Ges.*, 1876, ix, 217.
12. Scheitz, P., *Z. anal. Chem.*, 1910, xlix, 735.
13. Scheitz, P., *Z. anal. Chem.*, 1910, xlix, 736.
14. Treadwell, F. P., and Hall, W. T., Analytical chemistry, New York, 6th English edition, 1924, pt. 2.
15. Thorpe, E., Dictionary of applied chemistry, London, 3rd edition, 1922.
16. Clark, W. M., Determination of hydrogen ions, Baltimore, 2nd edition, 1923, 114.
17. Kolthoff, I. M., Der Gebrauch von Farbenindicatoren, Berlin, 1923.
18. Brode, W. R., *J. Am. Chem. Soc.*, 1924, xlvi, 523.
19. König, A., *Med. Ann.*, 1894, liii, 783.
20. Martens, F. F., *Verhandl. deutsch. physik. Ges.*, 1899, i, 280.
21. Sheppard, S. E., Photo-chemistry, London, 1914, 45.
22. Martens, F. F., and Grünbaum, F., *Ann. Physik.*, 1903, xii, 984.
23. Grünbaum, F., *Ann. Physik.*, 1903, xii, 1004.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

XI. THE INTRACELLULAR REACTION OF THE KIDNEY EPITHELIUM AND ITS RELATION TO THE REACTION OF THE URINE.

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In the past the injection of dye substances into the circulation of living animals has yielded much information about the functions of certain regions of the kidney (1-7). Recently Rous (8–11) has reported studies of the relative reaction of living cells carried out by the use of various indicator dyes. Some of his methods are well adapted to a study of the reaction within kidney cells and he has observed that litmus when segregated within the renal epithelium is in some places red, in others blue, a fact suggestive of marked functional differences. In the work here to be reported this observation has been followed up and we shall record certain changes in the reaction within cells lining the renal tubules of mammals, which changes accompany and are largely, if not entirely conditioned by alterations in the functional activities of these cells.

Previous Literature.

The older literature on vital staining of the kidney has been reviewed by Cushny (12) and recently by Marshall and Crane (13). The elimination of dye substances by the glomeruli has been demonstrated by Wearn and Richards (14–16) and by Bieter and Hirschfelder (17). Workers are at one concerning it, but there has been much difference of opinion as to the significance of the presence of dye substances within the tubule cells. Heidenhain (1) took their presence to indicate a removal of the dye by these cells out of the blood and into the urine, a secretory activity in other words; but many more recent workers have looked upon it (6, 12) as a result of absorption from the tubule lumen. Recently Marshall and Vickers (18) and Marshall and Crane (13) have reported studies of the excretion of phenol red which they take to indicate a direct secretion of the dye by the tubular epithelium.

Indicator dyes have been much used for the study of kidney function in general but very little for that of the reaction prevailing within renal cells. Long ago Dreser (19, 20) found acid fuchsin in the acid state in the cells of the convoluted and straight tubules of the frog following repeated injections of the dye, and Grützner (21) reported that the glomeruli were stained with indigosulfonate in the acid form under conditions of asphyxia. Rous (22) noted that the color of the renal cortex of rats and mice injected with various of the phthalein indicators was often such as would indicate a pronounced degree of acidity; and more recently Edwards and Marshall (23) have noted that phenolsulfonephthalein when present in the convoluted tubule cells of the dog kidney has the yellow color of acidity. Stieglitz (7) had previously employed azolitmin, neutral red, and sodium alizarinate in a study of changes in the reaction of the renal tissue. He injected the dyes directly into the blood stream of rabbits and dogs and killed the animals within a few minutes. He took the coloration to indicate that the reaction prevailing within the kidney cortex is alkaline when an acid urine is being secreted and *vice versa*. No histological evidence is given in his paper.

In the experiments here to be described we have studied the reaction within kidney cells by means of one of the pigmented ingredients of litmus, erythrolitmin (24–27), which is taken up and held by these cells for considerable periods. Rous, in his papers on the relative reaction of mammalian tissues (8–11, 22) noted that the tubule cells of the rat kidney, after an intraperitoneal injection of litmus, contained abundant granular deposits of the dye, which persisted for weeks and even months after the injection. Since the intracellular material was found on test to have retained its indicator properties it was evident that a study of the reaction within the cells could be made under varying physiological circumstances.

The Suitability of Erythrolitmin for Kidney Cell Studies.

More than 90 per cent of the purified "whole litmus" employed by Rous consisted of erythrolitmin, and it is to this ingredient that the staining he obtained is to be attributed. Erythrolitmin is far superior to azolitmin or to "whole litmus" for purposes of vital staining, being practically non-toxic, and its change from red to blue occurs within the range of hydrogen ion concentration encountered in acid and alkaline urines (12, 24). These facts, coupled with the finding that intraperitoneal injections of the substance did not cause nephritis, as evidenced histologically by sections of the kidney and by the absence of albumin in the urines of the injected animals, led us to employ

it for the present work according to a method described below. Only pure erythrolitmin was used, save where mention to the contrary is made. After intraperitoneal injections the dye is segregated within the tubular epithelial cells everywhere from the glomerulus to the collecting tubule, and shows striking regional differences in color under differing conditions of functional activity. A diffuse staining of the intracellular granules takes place, not a mere surface accumulation of dye such as some authors believe occurs (11) who have dealt with renal epithelium stained in other ways.

Character of the Staining.

The exact situation of dye substances segregated within cells has been the subject of great dispute. The indicator material retained within cells after injections of "whole litmus" is sometimes concentrated upon the surfaces of cell granules and in other cases is diffusely distributed within the latter (8, 9). The colored granules which can be squeezed from renal epithelial cells, after an intraperitoneal injection of erythrolitmin, show in our experience a diffuse distribution of the stain now in dilute form and again greatly concentrated. Many granules spread like masses of a soft gel when pressure is exerted upon the cover slip, but others resist such pressure as can readily be brought to bear and retain their form. There are never any appearances that would suggest a precipitation of the dye. Always it is evenly distributed and the granules are translucent.

Procedure.

Rats of 80 to 200 gm. weight were given intraperitoneal injections of a 2 per cent solution of erythrolitmin in 0.9 per cent sodium chloride solution. The material had been prepared according to a method described in the foregoing paper (24). For every gm. of body weight $\frac{1}{4}$ to $\frac{1}{2}$ mg. of the dye was given and the injection was repeated once or twice at 48 hour intervals. The result was an excellent staining of the kidney. As a rule the organ was examined 48 hours after the last injection, but the findings were much the same when the interval was several hours shorter. At the time for examination of the kidneys the animals were given a whiff of ether, guillotined, stretched out upon a paraffin slab, and submerged in paraffin oil. The kidneys were removed with all possible speed, decapsulated, and sectioned with a Valentine knife while still under the oil. The whole process required often no more than 45 seconds to 1 minute. The sections, protected by oil, were placed on mica slides, covered with thin slips of the same material, and

ringed with paraffin. As these sections were often about 100μ thick, and on the average about 150 to 175μ, observations on the color of the dye granules within the kidney tubule cells could readily be made. Frozen sections also were made and studied to determine the distribution of the dye irrespective of its color. On freezing the tissues, the granules previously diversely colored, red, blue, or violet, became all of one hue, violet, as result of the process of freezing and immersion of the sections in water before mounting. The granules within the cells of fresh sections made with the Valentine knife, on the other hand, did not change color during the first few minutes of observation, often maintaining their original hue for 20 minutes or more, a fact which may be taken to indicate that the coloration was identical with that existing during life.

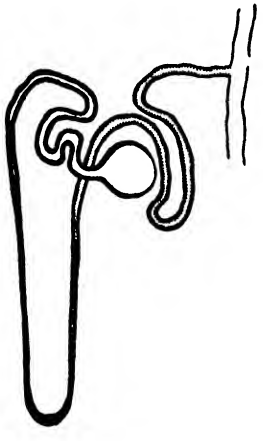
Findings in the Normal Rat Kidney.

In the gross the stained kidneys appeared a dark purple-red. Under the low power of the microscope the outer zone of the cortex appeared blue at first sight, the inner zone red, and the medulla unstained. Closer inspection showed, however, that there were marked local differences underlying the prevailing hues. Much of the renal cortex was unstained. The glomerular tufts showed no litmus but were well filled with blood cells of the natural hue. The endothelial lining cells of the glomerular capsule and particularly those near the entrance of the uriniferous tubule always appeared a deep brilliant blue, containing the dye either in very fine granules, or at times in what seemed to be the diffuse form. Wherever the proximal convoluted tubule could be seen leading off, the cells lining it contained the dye in bright blue granules, which were so abundant as to lend a blue hue to the cells. In most sections this picture was readily to be observed. The glomerular tufts appeared as though in the cup of a blue crescent with a blue stem.

Wherever the eye could follow a single tubule far toward the renal pelvis, the color of the granules contained in the epithelial cells was observed to vary with the region. Near the glomerulus the color was blue, and further down violet, while it was red a very little further on. In that short segment where the tubule cells in general appeared violet, owing to their content of granules having this color, individual epithelial cells were to be found containing granules some of which were brilliant red and others bright blue. In every section there were portions of tubules occupying the zone intermediate between the outer and inner cortical areas of the kidney, that could not be identified definitely. These usually contained violet granules.

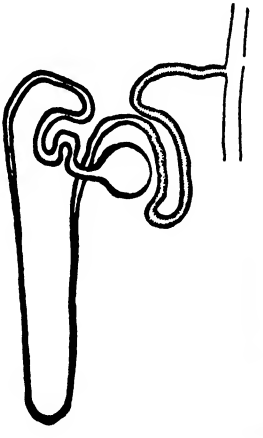
In the inner zone of the renal cortex were to be seen in every section many characteristic loops of Henle, mostly of a brilliant blue, but some violet, and a few red. Adjoining these portions bits of tubules were often made out from just above or just below the loops of Henle in which a transition from blue through violet to red or *vice versa* could be seen. The above mentioned unidentifiable bits of tubules containing the dye in violet form may be considered to have come from regions lying a little way above or below the loops of Henle.

In the outer cortical region convoluted tubules could be seen the cells of which all contained the dye in red granules. In no instances were the striations, characteristic of proximal convoluted tubules, present among them. Very often these "red tubules" emptied directly into the collecting tubes, in which case there was, of course, no doubt that we were dealing with distal convoluted tubules. In striking contrast, the proximal portions of the convoluted tubules, when definitely identified, either by their striations or when emerging from a glomerulus, contained the dye in the blue form. Never was any dye to be seen in the cells of the



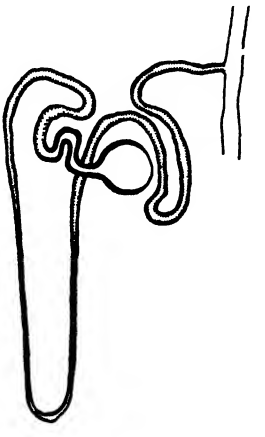
Neutral urine

TEXT-FIG. 1.



Alkaline urine

TEXT-FIG. 2.



Acid urine

TEXT-FIG. 3.

Text-fig. 1 shows diagrammatically the renal color pattern following intraperitoneal injections of erythrolitmin in rats secreting neutral urine. Text-figs. 2 and 3 show the changes in color pattern associated with the secretion of alkaline and acid urine, respectively, described in the text. The region of blue erythrolitmin is indicated by solid black, that of violet by dots, and of red by the short lines.

collecting tubules and none was ever recognizable in the glomeruli or tubular lumena.

Sometimes all of the findings described could be made out in a single preparation. From the examination of scores of fresh sections, from many animals, we have prepared Text-fig. 1, which shows diagrammatically the state of affairs in the kidney of a rat maintained on an ordinary diet of barley, oats, and bread and milk, and excreting a very slightly acid or a neutral urine.

In brief, then, during the excretion of neutral or very slightly acid urine, the granules of the lining cells of the glomeruli as well as those

of the cells of the derivative tubules, at times as far along as the loops of Henle, are alkaline to erythrolitmin. Further on, in a short segment of tubule the cell granules show the violet color of neutrality, while further yet, in the cells in the ascending limb and in the distal convoluted tubules, the granules are acid in reaction (Text-fig. 1). There is no dye anywhere to be seen in the lumina of the tubules and none within the cells of the collecting system.

The Effect of Diuresis on the Staining with Erythrolitmin.

A number of rats were provided for several days with nothing but a 10 per cent watery solution of cane sugar which they drank in great quantities. During this period erythrolitmin injections were given as in the instances described above. A series of control animals fed on a diet of bread and milk, barley and oats and injected with similar doses of erythrolitmin, were employed for comparison. The urine secreted by the sugar-fed animals appeared slightly more alkaline to brom cresol purple and phenol red than did that of the controls and was from two to four times as abundant. In the kidneys of individuals killed at the height of the diuresis, sections of the renal tissue, viewed in the gross, appeared to contain far more blue dye. Microscopically, fewer unstained areas were seen and the loops of Henle were oftener of a bright blue, though the cells of the distal convoluted tubules were still always red. The point of change from the blue to the red reaction was apparently situated further down the tubule.

The experiments suggested that with an increase in the quantity of the urine and a change to slightly greater alkalinity, more, relatively speaking, of the litmus was present in the alkaline form.

The Kidneys during the Excretion of Alkaline Urine.

Animals to which sodium bicarbonate had been given were now studied.

Rats weighing from 75 to 200 gm. were given by stomach tube, in the morning and again late in the afternoon of the same day, 5 cc. of a 10 per cent solution of sodium bicarbonate. A plentiful excretion of alkaline urine always resulted. On the 2 or 3 days following, during which the bicarbonate administration was continued, injections of a 2 per cent erythrolitmin solution were made as in the

normal control rats. There were either two or three injections, on successive or alternate days. After a final lapse of 48 to 72 hours, without discontinuance of the bicarbonate administration, the animals were killed and the kidneys examined microscopically. To vary the conditions some individuals, previously injected twice or thrice with erythrolitmin, were given the alkali once, then again on the following morning, and in the afternoon they were killed and studies of the kidneys were made. The findings were the same whichever the procedure.

In the gross the kidneys appeared deep purple-red as ordinarily. Microscopically the general color of the cortex was blue, but scattered areas of cells with brilliant red granules were to be found. As usual the glomeruli lay within blue crescents of endothelial cells packed with blue dye, and the capillary tufts were filled with blood of natural color. The cells of the proximal convoluted tubules leading away from the glomeruli were crowded with granules of a dense deep blue. Nearly all the tubular loops which could be identified as descending limbs of the loops of Henle were deep blue and so too with the ascending limbs for some distance. In the cortex, however, many red tubules were seen, the cells thereof containing in no instance the striations of proximal convoluted tubules. A connection of these red tubules with the collecting tubes could frequently be made out and hence they must have been portions of the distal convoluted tubules. But segments of them,—not adjacent to the collecting tubes but lying further up,—were blue or violet, a phenomenon never observed in the animals secreting neutral urine. The collecting tubules themselves were unstained.

A change in the relative reaction within the tubule cells as evidenced by the distribution of the litmus colors had evidently taken place in these animals. The blue coloration extended much further down, being seen in the granules of the cells of the ascending limb of the loop of Henle and in an adjoining portion of the distal convoluted tubules. In every case the terminal portions of these latter where they joined to the collecting tubules were red with the indicator, as under normal conditions, and this despite the fact that a markedly alkaline urine was being secreted.

The findings described above have been gathered from an examination of many sections from many animals, though occasionally a single specimen showed them all. By and large they demonstrate clearly that, during the secretion of alkaline urine, the reaction within the cells of the kidney cortex is markedly altered. The essential difference from the normal lies in an extension of the blue color along the tubular path, in some instances almost as far as the collecting tubules. Text-fig. 2 shows this diagrammatically.

Appearance of the Kidneys during the Secretion of Acid Urine.

Rats injected with erythrolitmin were caused to secrete acid urine. The kidneys showed changes the opposite of those occurring when the urine had been rendered alkaline.

The secretion of acid urine was brought about in rats by the administration of a 10 per cent aqueous solution of sodium acid phosphate through a stomach tube. At the same time control animals were fed ordinary food and other rats were fed alkali for the experiment already described. Rats of like age and weight were chosen for the procedures. Half of those given sodium acid phosphate received two doses in a single day and 24 hours later the first of the injections of erythrolitmin. Either two or three injections of the dye were made, on alternate or successive days, and the examination of the kidneys was carried out 48 hours after the last injection. Throughout the period of preparation, 5 to 8 days in all, the feedings of sodium acid phosphate were continued; and the animals' urine was regularly acid during the period. The other half of the rats were fed acid solution for the first time on the day after the last of several erythrolitmin injections, and again on the following morning. 6 hours later the kidney examination was made, at a time when acid urine was being formed. In both groups the findings were the same.

In the gross the kidneys appeared reddish purple. Microscopically the sections appeared almost entirely a bright red in contrast to the spotty red and blue of the controls. The outer areas of the cortex were red, with only here and there tubules containing the dye in blue form. The tubules of the inner cortical area were all red, including the loops of Henle. But it was striking that even in these kidneys the glomeruli lay cupped in blue crescents just as in the controls, the endothelial lining cells containing erythrolitmin only in the blue form. Wherever a tubule could be found leading off from the tuft the cells near the glomerulus likewise contained blue granules. Text-fig. 3 shows diagrammatically the findings in these experiments. The cells of the first portions of the proximal convoluted tubules contained the dye in the blue form only, but the changes from blue to violet and then to red occurred close to the glomerulus, considerably nearer than in normal animals, and far nearer than in the kidneys of the rats fed alkali. Frequently the shift in color of the intracellular granules was seen to occur at a point nor more than 100 or 200 μ from the exit of the tubule from the tuft. In numerous instances the eye could trace the entire color sequence within a relatively short extent of tubule. Many individual cells in the region of color change where the granules were preponderantly violet contained both bright blue and brilliant red granules lying together in the cytoplasm. Distal to the region of change all of the intracellular granules were red. The few blue areas in the cortex could always be identified as segments of the proximal convoluted tubules from next the glomerulus. Cells containing dye in the blue form were never found further along. Brilliant

red areas were frequent that consisted of proximal convoluted tubules. No blue loops of Henle were seen; all were red. As in the other sections the collecting tubules were quite without color.

To sum up the alteration in the reaction within the tubule cells, as evidenced by the litmus change to red, affected the proximal convoluted tubules throughout almost their whole length and the entire loop of Henle. The structures mentioned now showed acidity instead of the alkalinity which is normal to them.

Constancy of the Findings.

The phenomena described occurred with but very little individual variation. Certain of them were constant. No matter what the reaction of the urine the granules of the cells lining the glomerulus and the adjoining portion of the proximal convoluted tubules were blue. And the intracellular granules of the further portion of the distal convoluted tubules were always red. Erythrolitmin was stored throughout the system from glomerular tuft to collecting tubule but not in either of these structures. In normal animals secreting a neutral or slightly acid urine the transition from red granules to blue, the zone that is to say where the granules are predominantly violet, is situated in the beginning of the ascending limb of the loop of Henle.

The relative reaction of kidney cells and their changes under functional conditions were studied in some other species besides the rat. Mice, guinea pigs, and rabbits were given intraperitoneal injections of erythrolitmin in dosages similar to those used in the rat experiments, that is to say $\frac{1}{4}$ to $\frac{1}{2}$ mg. of the dye per gm. of body weight. The secretion of acid and alkaline urines was induced in the way already described, by feedings of sodium acid phosphate or sodium bicarbonate; and the state of the kidneys was compared with that of normal animals injected with erythrolitmin. The findings were altogether similar to those in the rat, so they need not be enlarged upon.

Constancy of the Findings Following Massive Doses of Acid and Alkali.

Acid Administration.—A remarkable feature in the experiments just described was the regular occurrence of erythrolitmin in the blue form

TABLE I.

Rat No.	Body weight	Daily administrations of HCl (concentration and amount)	Character of secretion of urine	Urine pH	Interval between last acid dosage and autopsy	Color of erythrolitmin in glomerular lining cells		
	gm.				*hrs.*			
1	142	1 of N/10, 10 cc.	Copious secretion	6.0	16	Blue		None red
2	150	4 of N/10 @ 10 cc.	Copius secretion	5.6	16	Blue		None red
3	122	2 of N/10 @ 10 cc.	Few drops only on 2nd day	6.0	5	Blue	Some blue-purple	None red
4	128	1 of N/6, 5 cc.	Copious secretion 6 hrs .later	5.9	6	Blue	Some blue-purple	None red
5	125	1 of N/3, 5 cc., followed by water, 9 cc., in 1 hr.	Scanty secretion 2 hrs. later	5.4	2	Many blue	Many purple	None red
6	135	1 of N/4, 9 cc.	Scanty secretion 6 hrs. later	5.5	6	Many blue	Many purple	None red
7	99	1 of N/4, 10 cc.	Very scanty secretion 5 hrs. later	5.9	5	Many blue	Few purple	None red
8	104	" "		to		Change from blue to red in proximal convoluted tubules very close to glomerulus		
9	111	" "		6.2				
10	106	1 of N/4, 10 cc.	Hematuria 2 hrs. later. Suppression 5 hrs. later		5	Very few bluish purple	Mostly purple	A few red
11	104	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.5 to 6.0	5	Few blue	Many purple	None red
						In proximal convoluted tubules cells near glomerulus purple to red		
12	129	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.5	5	Many purple		Red in two glomeruli

13	120	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.0	5	None really blue		Several red-lavender
14	110	3 of N/10 @ 10 cc. 1 of N/2, 2 cc.	Last day suppression		16	None blue		Many red
15	154	3 of N/10 @ 10 cc., a 2 day interval, then 1 of N/2, 2 cc., then 1 of N/4, 10 cc.	Last day suppression		7	None blue	Many purple	Some red
16	101	1 of N/3, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later hematuria 6 hrs later suppression		6	Few blue	Mostly purple	Some red
17	109	1 of N/3, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later suppression 4 hrs. later suppression		4	None blue	All lavender	None red
18	122	1 of N/2, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later hematuria 5 hrs. later suppression		5	Few blue	Mostly purple	Some red
19	168	1 of N/1, 14 cc.	1½ hrs. later hematuria 3 hrs. later suppression		3	Few blue	Mostly purple	A few red

within the cells lining the glomerulus and the adjoining portion of the proximal convoluted tubules, no matter what the reaction of the urine that was being secreted. Litmus segregated within healthy cells elsewhere throughout the body is regularly in the acid form, red that is to say. It was deemed worth while to test whether the blue of the glomerular litmus is changed to red by massive doses of acid.

For the purpose, rats weighing from 99 to 168 gm. were given dilute HCl by stomach tube in varying concentrations and amounts, as outlined in Table I. As a rule, several administrations of HCl were employed at 24 hour intervals, or a single dose in higher concentration. The animals were kept in special cages for the collection of urine under oil. Fresh bladder urine unmixed with feces was obtained for pH estimation with indicators by holding a closed cone over the animal's nose for a moment, thus inducing a struggle during which urine was voided. In all other respects the technique was that already described.

Table I summarizes the result of this experiment. The animals receiving the smaller quantities of acid (Nos. 1 to 9, inclusive) continued to secrete urine. In the kidneys of these rats erythrolitmin, present in the lining cells of the glomerulus and epithelial cells of the first portion of the proximal convoluted tubules, was still always blue. In both of these regions it frequently appeared bluish purple, but never frankly red. The color change of the dye in the cells of the proximal convoluted tubules took place much closer to the glomeruli than in the earlier experiments, often less than the distance of one glomerular diameter away from the exit of the tubule from the tuft.

Suppression of urine, usually preceded by hematuria, followed the large quantities of acid given to Animals 10 to 19 , inclusive (Table I). In contrast to the findings of the foregoing experiments, erythrolitmin appeared at times in the red form within the glomerular lining cells and those of the first portion of the proximal convoluted tubules. But only after urinary suppression could this be demonstrated. Whenever secretion continued (Rats 1 to 9 inclusive), the dye in the locations named remained either blue or a deep bluish purple. A comparison of the findings of Rats 7, 8, and 9 with those of 10 shows this point well, for all these animals received similar amounts of acid but with differing results. So, too, does a comparison of the findings of 3 with those of 11, 12, and 13.

The effect of massive doses of acid may also be evoked by means of an "acid-forming" diet. Feedings of $CaCl_2$ have been shown by Haldane, Hill, and Luck (28) to have the effect of administrations of HCl; for the calcium moiety is not retained, while the chlorine is. According to Gamble, Ross, and Tisdall (29), 40 per cent of the chlorine given in this way behaves in the body like HCl. As Addis, MacKay, and MacKay have pointed out (30), a rat eating 10 gm. of diet containing 4 per cent of $CaCl_2$ should receive the equivalent of 14.4 cc. N/10 HCl.

We therefore maintained rats for 10 days on a diet of bread and milk to each 100 gm. of which 4 to 6 gm. of $CaCl_2$ were added. Eight of the animals were

injected with erythrolitmin on 2 alternate days and killed 48 hours after the last injection. The special diet was continued to the end. Urine was secreted plentifully and of a pH usually well below 6.0. Upon examination of the kidneys the dye still appeared blue in the glomerular lining cells and the first portion of the proximal convoluted tubules.

Alkali Administration.—Massive doses of alkali were given to rats stained with erythrolitmin, to see whether in this way the color of the dye present in the lining cells of the final portions of the distal convoluted tubules could be influenced. Even after the feedings of sodium bicarbonate outlined earlier in this paper it had been constantly in the red form.

Stained rats were given by stomach tube 4 cc. or 2 cc. of N/1 Na_2CO_3 solution per 100 gm. of body weight. The larger doses led to rapid suppression of urine, the smaller often to a similar result after 48 hours. The urine when obtained was usually of a pH about 8.2, as determined by the indicators cresol red and thymol blue. Examinations of the kidney were made, in many instances, before this result had been attained, and in others afterwards. We were never at any time able to demonstrate the dye in the blue form within the lining cells of the final portion of the distal convoluted tubules where these were actually observed emptying into the collecting tubules. The dye was frequently of a reddish lavender but never frankly blue. In all other situations throughout the tubular epithelium, it was intensely blue.

It is evident that massive doses of acid fail to change from blue to red the erythrolitmin segregated in the cells lining the glomeruli and the first portion of the proximal convoluted tubules. As long as the kidney continues to secrete urine these cells maintain a relative alkalinity, despite the strong acidity of the urine. Only in the event of urinary suppression is there some slight shift in the direction of acidity.

After massive doses of alkali, sufficient sometimes to cause urinary suppression, the erythrolitmin within the cells lining the final portion of the distal convoluted tubules remains red.

Results after Intravenous Injections.

To complete our observations some intravenous injections of erythrolitmin were made. The method has already been used by Stieglitz (7) who employed azolitmin, neutral red, and sodium alizarin upon rabbits and dogs.

Rats of approximately 100 gm. weight were injected intravenously with 1 or 2 cc. of a 1 per cent solution of erythrolitmin at a pH of approximately 7.0. The kidneys were extirpated and sectioned after intervals of 30 minutes, 1 hour, 2, 5, 6, 10, and 18 hours. The animals tolerated the dye poorly and in many instances became moribund a few minutes after the injection.

The findings were by no means as clear-cut as after intraperitoneal injection of the indicator since the kidneys never became well stained.

Within a few seconds after intravenous injection of erythrolitmin the urine contained it in high concentration whereas the kidney tissue showed not the slightest traces. When half an hour had elapsed the glomeruli appeared faintly bluish regardless of the reaction of the urine. The color seemed to be due to the presence of the dye in the blood within the tufts. When 2 hours had elapsed some of the tubular epithelial cells of kidney sections showed a very faint diffuse blue tint. No definite coloration of intracellular granules could be made out, and the staining was so slight that it appeared merely as a blue haze over the cells. 6 to 8 hours after an injection of the dye, colored granules could be found here and there in the tubule cells. Both red and blue granules were to be found, indiscriminately located. In this connection it may be recalled that litmus is often taken up by cells in fine blue particles and only secondarily converted to red (9) as further that when a cell containing segregated litmus is damaged or has begun to degenerate it turns from red through violet to blue (9). The coloration of the kidney cells was so poor, as compared with that obtained after intraperitoneal injections, that no conclusions as concern kidney function could be drawn from the instances mentioned.

With a further increase to 24 or 48 hours in the time interval between the injection of erythrolitmin and the examination of the kidney, the appearance of the organ came to resemble closely that following intraperitoneal injections. Nothing, then, had been gained by the intravenous administration of the indicator.

We were unable to secure satisfactory staining of the rat's kidney after intravenous injections of azolitmin (Grübler), with our own preparation of azolitmin or with sodium alizarin VI, dyes with which Stieglitz (7) reported success in dogs. Exitus frequently occurred soon after injection of the dyes when given in quantities too small to stain the kidneys.

It has been reported by Stieglitz that, after the intravenous injection of azolitmin, sodium alizarin, and neutral red, the reaction of the kidney cortex is apparently the opposite of that of the urine secreted at the time. In view of our failure to confirm this finding with the aid of the dyes just mentioned it seemed necessary to repeat Stieglitz's experiments with neutral red.

Solutions of neutral red in physiologic saline when given intravenously stained the kidney well in dogs and rabbits within a few minutes. The tubule cells took up the dye in both a diffuse and granular form and it appeared promptly in the urine. However, under these circumstances there was no marked differential staining, as with erythrolitmin. The kidney was always of an almost uniform hue, which hue depended largely, as will be shown, upon the dosage of the dye and the time interval between its injection and the examination of the organ. In passing it should be said that the yellow, alkaline form of neutral red can easily be confused with the natural yellowish color of thin sections of kidney tissue.

Six dogs and 16 rabbits were induced to secrete alkaline or acid urine, as desired, through the oral administration of sodium bicarbonate or sodium acid phosphate, or by the intravenous injection of these substances. After the bladder urine obtained by catheterization had become definitely alkaline or acid, intravenous injections of neutral red (Grübler) were given, in 1 per cent or 0.5 per cent solution in physiological saline. Various doses were used and the kidney examined after various intervals of time. Specimen protocols follow:

Normal dog, 8½ kilos.

10. 50 a.m. Ether anesthesia induced; the bladder urine is distinctly acid to brom cresol purple and neutral red. 10.52. Catheterization; urine specimen acid as before; catheter bound in.

10.55. *30 cc. of 1 per cent solution of neutral red (Grübler) in 0.9 per cent NaCl solution injected intravenously.* The animal became rose red at once. 11.01. The dye appeared in the urine, in the red form. 11.25. The right kidney was removed through a posterior incision. In the gross it appeared deep red. In sections, cut with a Valentine knife and mounted between mica slides, the tubular epithelial cells were colored red, and many contained the dye in granular form. The general staining of the kidney, however, was slight.

11.30. *Intravenous injection of 5 per cent solution of sodium bicarbonate, 150 cc.* 11.33. The urine became orange. 11.40. The urine was clear and dark yellow; it turned red upon the addition of acid. 12.00 m. The left kidney was removed. In the gross the whole organ appeared yellowish. Sections, made with the Valentine knife and mounted as described above, appeared yellow. The tubular epithelial cells were stained diffusely yellow.

In this experiment the kidney removed after the animal had begun to secrete alkaline urine appeared definitely more alkaline than the organ examined at a time when the urine was acid.

In another experiment after an intravenous injection of 5 per cent sodium bicarbonate with consequent elimination of alkaline urine, a larger intravenous injection of 1 per cent neutral red was given, 45 cc. to a 9 kilo dog, and the kidneys were removed 12 minutes later instead of after half an hour, as in the experiment above. Microscopically the epithelium of the renal tubules appeared red although the urine secreted was yellow, a finding the opposite of that just described, but in agreement with the experiments of Stieglitz (7). The test was repeated on

another 9 kilo animal with the same dosage of dye but one of the kidneys was removed half an hour after an alkaline reaction of the urine had been established, instead of after the lapse of but 12 minutes as in the previous experiment. The second kidney was removed 30 minutes after an acid reaction of the urine had been established. With this greater interval of time between the injection of dye and the examination the color of the kidney cortex was found similar to that of the urine secreted. The protocol follows:

Normal dog, 9 kilos. All urine specimens obtained by catheter.

10.45. a.m. Ether anesthesia. 10.50. Urine clear and strongly acid to neutral red, erythrolitmin, and brom cresol purple.

10.58. *Intravenous injection of sodium bicarbonate, 5 per cent solution, 75 cc.* 11.08. Intravenous injection of neutral red, 1 per cent, 40 cc. 11.14 (6 minutes later). Urine appearing red from the catheter,—still acid. Intravenous injection of sodium bicarbonate repeated. 11.17. Urine bright orange, that is to say definitely alkaline. 11.47. Urine has been alkaline for ½ hour. Right kidney removed. Histological study made at once (see below).

11.49. *Intravenous injection of sodium acid phosphate solution, 5 per cent, 70 cc.* 11.51. Urine now reddish rose colored. 11.53. Injection of sodium acid phosphate repeated. 11.55. Urine bright rose red, strongly acid. 12.21 p.m. Urine has been acid for ½ hour; left kidney removed. Histological study made at once. Animal killed with chloroform.

The kidneys were removed, in each instance 30 minutes after the urine had been rendered acid or alkaline, respectively. When the animal was passing yellow alkaline urine the gross appearance of the kidney was a reddish yellow. Microscopically, sections mounted on mica slides and ringed with paraffin showed a moderate, diffuse, and granular staining of the cortex. The dye in the tubule cells was mostly in the yellow form, but some in the red. The predominating color, though, was yellow, like that of the urine.

When acid urine was being secreted the kidney appeared bright red in the gross. Nearly all the dye in the epithelium of the tubules was present in the red form, with here and there a little yellow coloration. Again the predominating color of the cortex was like that of the urine.

Such experiments were many times repeated, with different doses of neutral red. The dye was given in the red form, but was brought close to the neutral point with sodium bicarbonate. It cannot be injected in the yellow form because of the resulting precipitation of the dye in alkaline reaction. In general, when large doses of the dye were given and examination of the kidney made shortly afterwards, the tubule cells contained much of it in the red form, regardless of the reaction of the urine. When half an hour or more was allowed to elapse between the injection of the dye and examination of the kidney,

and when the dosage of dye was not too great, the majority of epithelial cells manifested the same color as did the urine.

Similar experiments upon rabbits gave findings of like sort. When large doses of neutral red were employed and the examination made soon thereafter, the kidney cortex, in general, appeared red, no matter what the reaction of the urine. Lengthening the interval between injections of the dye and examinations of the kidney gave time for the organ to convert most of it in the tissues to the form in which it appeared in the urine.

The protocol which follows contains the findings in four rabbits, and shows well the influence of dosage, and time interval, upon the coloration of the kidney cells with neutral red.

The treatment of the animals may be tabulated as follows:

Rabbit	*a*	*b*	*c*	*d*
Weight	650 gm.	655 gm.	647 gm.	653 gm.
Intravenous injection of 5 per cent sodium bicarbonate	8 cc.	8 cc.	8 cc.	8 cc.
5 min. later. Intravenous injection of 1 per cent neutral red	12 cc.	2 cc.	12 cc.	2 cc.
Kidney examination	5 min. later	5 min. later	90 min. later	90 min. later

After the bicarbonate injection the urine of all the animals was strongly alkaline and contained much dye in the yellow form.

Rabbit a.—In the gross the kidney appeared dark red. Section showed the tubule cells containing much red dye, in granular as well as diffuse form. The cortex was predominately red.

Rabbit b.—The kidney appeared red in the gross, but lighter in color than that of Rabbit *a*. Sections were yellowish red. The tubule cells contained dye in red granules. The general appearance of the kidney of *a* was more acid than that of *b*. N/50 HCl run under the cover-glasses turned the sections as a whole a deeper red, showing that some of the dye was present in the yellow form.

Rabbit c.—The kidney appeared a mottled reddish yellow. The sections were red in general and deeply stained. Many tubule cells held the dye in red granular form, while in other tubules the cells contained yellow granules. The glomeruli appeared as distinct yellow crescents. The urine was a deep yellow. The

bladder, however, contained yellow urine with clot-like masses of dye in the red form. When the contents were mixed the prevailing hue was yellow.

Rabbit d.—In the gross the kidney appeared yellow. Sections were also yellow. There was more dye present in the tubule cells than in those of Rabbit *b* which received the same dose of the indicator, and less than in Rabbits *a* and *c*. Practically all of it was present in the alkaline form.

From the results of these and other like experiments certain facts became plain. When the dose of neutral red was small the tubular epithelium of the kidney usually appeared of the same color as the urine secreted, though in a few instances it had the opposite color. When the dose was large enough to stain the tissue well much of the indicator within the cells manifested the color it had when injected, regardless of the reaction of the urine. When the time interval between the injection and the examination of the kidney was short, that is to say half an hour or less, the cells might or might not have the same color as the urine. If the time interval was lengthened, the color of the majority of the kidney cells was that of the urine being secreted. Apparently the lengthening of the interval between the intravenous injection of a large amount of dye and the histological examination gave time for its conversion in the stained tissues to the form in which it appeared in the urine. If the time interval after injection was short, it seemed largely a matter of chance how the kidney was stained owing to the great concentration and overwhelming amount of material suddenly brought to it.

Altogether it is obvious that the results of the intravenous method of administration of indicator dyes for renal staining are untrustworthy, unless examination of the organ can be postponed until the cells can deal with the material coming so suddenly to them. In practice many indicator dyes are toxic, when administered in this way, and the animals frequently die before sufficient time for a proper staining of the tissues has elapsed.

DISCUSSION.

Erythrolitmin is far from being a perfect indicator (24). In view of this fact how much is one justified in inferring from the present work? The color of most indicators varies with their concentration

and erythrolitmin is no exception to the rule. There is then a definite, though slight, increase in the red in higher concentrations; and since erythrolitmin would appear to be often very highly concentrated when segregated within the kidney tubule cells, the possibility of an interpretative error from this source may be great. The dye is subject to large salt and protein errors as well, but these influence it in the opposite direction. Erythrolitmin in the presence of salt or protein at a given pH appears more blue than in solutions at the same pH without these ingredients (24).

Granting these facts erythrolitmin still has notable virtues as an indicator of tissue conditions. It persists within the tissues for months, eliciting no evident reaction and retaining its indicator characteristics. This is true of the dye segregated within renal epithelium. When a section of the stained kidney is placed in a buffer solution all the stained granules take the same color, namely one approximating that which would be expected of erythrolitmin at the pH of the buffer. In untreated specimens immediately after removal from the body many granules containing the dye in highly concentrated form are a deep blue. Since the optical error upon concentration is toward the red, it is evident that this factor plays no decisive part in the color.

Can the findings of the experiments outlined in the present paper be due to supravital changes? The constancy of the results under a given set of conditions and the equal constancy of the induced variations would seem to put this possibility out of the question. One might suppose that the knife in sweeping from cortex to medulla and carrying with it a mixture of blood, lymph, glomerular filtrate, and more or less completed urine would bring about an alteration in the granules of the cells with which it came in contact. So it must in those cells that are injured. Such an effect will not explain, however, the orderly arrangement of red and blue granules everywhere in the unexposed cells. These cells show no change in the litmus coloration for many minutes after removal from the body. The kidneys can be removed from living animals, sectioned, and observed, within 45 seconds to 1 minute. Renal cells can scarcely be regarded as dead in so short a time, much longer interruptions of the circulation being withstood (31).

Can one suppose the variations in the reaction of the granules in the living cells of the renal tubules to be a mere passive reflection of the reaction of the fluid passing along the tubules? Such a view is incompatible with what is known of cell integrity. Litmus segregated in granular form by phagocytes does not take the color appropriate to the hydrogen ion concentration of the fluid surrounding these cells unless they are crushed or otherwise injured (9).

All things considered, one is forced to the conclusion that the color patterns seen in kidneys stained with erythrolitmin are the expression of functional activities. But how these activities exert their influence on the reaction of intracellular granules is a problem of great complexity.

The fact has already been emphasized that the granules within the epithelium of the glomerulus and the adjoining portion of the convoluted tubules are regularly blue, no matter what the reaction of the urine. Were it not for the cell integrity just mentioned the finding might seem explicable on the basis that the renal filtrate as derived from the blood and bathing these cells is ordinarily alkaline. Everywhere else though throughout the body litmus-containing elements are bathed by alkaline fluids yet the color of the indicator within them is red, indicative of acidity. Why should there be this peculiar exception in the case of this region of the kidney? And what is one to think of the shifting in the renal color pattern that can be brought about by administering substances to the animal which cause the reaction of the urine to shift to the alkaline or acid side. The tubule cells adjoining Henle's loop constitute, as it were, a transitional zone of color change. In them, erythrolitmin granules are violet, under normal circumstances, or sometimes red and blue in the same cell. During the secretion of acid urine the cells of this region contain the dye in the red form. One might suppose the red of the granules in this region to be of no greater significance than that met elsewhere in the body were it not for the fact that the red can be altered to blue by altering the functional conditions, as during the secretion of alkaline urine. One can only suppose these phenomena to be an indirect expression of functional activities on the part of the renal cells—cells through which material is passing into or perhaps out of the blood. Only in the case of the lining of the distal convoluted tubules where these join the collecting

system is another explanation possible. Through thick and thin, so to speak, the litmus within such cells remains in the red form, the form in which it is encountered in healthy, non-secreting cells elsewhere in the body. Here there would seem to be no reason to invoke a functional passage of material through the cytoplasm in order to explain the findings. Since the reaction within these cells cannot be rendered alkaline by any of the experimental means as yet attempted, it is conceivable that it is held fixed, as is certainly not the case higher up. Yet since these cells differ in no wise morphologically from those immediately adjacent to them in the same tubule, and do differ sharply from the elements of the collecting tubules upon which they abut, it seems more likely that they are protected by a fortunate situation from those functional stresses which in our experiments led to a change in the reaction of the granules further up.

Granted that the renal color pattern and its changes are the expression of functional activities, what is the nature of these latter? Do the cells lining the tubules resorb fluid only, or secrete materials into the tubular lumen as well? Unfortunately the findings here described would appear to admit of both possibilities. One can say at the moment only that the reaction within the cells of the renal tubules varies from region to region in a way which shows it to be intimately connected with the secretory activities of the organ. In some regions it is profoundly altered by changes in these activities.

Stieglitz' (7) conclusion that the reaction of the kidney cortex is the reverse of that of the urine being secreted at the time is based on observations of the cortex color following intravenous injections of indicators. Our repetition of some of his experiments has shown that the color of the kidney cortex shortly after such injections is influenced chiefly by factors not considered by Stieglitz, namely the dosage of the dye and the variations in the time interval between its injection and the examination of the organ. Large amounts of indicator dye coming suddenly to the kidney from the blood may be found, a few minutes later, unchanged in color within the cortex cells regardless of the reaction of the urine. The kidney overwhelmed, so to speak, with dye requires an appreciable time to act upon all the indicator substance present. A heavy dose of neutral red, in the acid form, injected intravenously into animals secreting alkaline urine, will appear in the

kidney cortex a few minutes later still in its red, acid form, indicating a reaction the reverse of that of the urine being secreted at the time. Later the dye in the cortex will be in the yellow, alkaline form. Small doses of neutral red intravenously injected in the acid form into animals secreting alkaline urine are of course converted to the alkaline form in the kidney sooner than are large doses. When acid urine is being secreted, neutral red injected in the acid form remains unchanged regardless of the dosage given.

Such findings clearly show how the employment of indicators by intravenous injection may lead to erroneous conclusions concerning the relation between the reaction of the kidney cortex and that of the urine secreted. They show further that the examination should be postponed until the cortex cells have had ample time to deal with the dye coming to them. Under these conditions one finds the reaction of the kidney cortex to be, by and large, the same as that of the urine under secretion.

SUMMARY.

The tubules of the mammalian kidney, vitally stained with erythrolitmin, show a significant color pattern, the cells of certain regions appearing bright blue and others brilliant red. The dye is segregated within the cytoplasm, staining fine granules diffusely.

Under normal circumstances of renal function erythrolitmin is stored in the lining cells of the glomerulus and the epithelial cells of the proximal convoluted tubules in the blue, alkaline form. In the cells of the final portion of the distal convoluted tubules the dye is red. Between these regions a narrow transitional zone can be found, at times above, at times below the loop of Henle, in which the erythrolitmin-stained granules in the tubule cells are violet, or red and blue within the same cell.

Alterations in the relative reaction of certain regions of the tubules, as disclosed by the color of the dye within the cells, can be induced by means that alter the reaction of the urine. When acid urine is being secreted there is a change from relative alkalinity to acidity high up in the tubular canal, in the proximal convoluted tubules. During the secretion of markedly alkaline urine the intracellular granules appear blue most of the way down the tubular canal, even to the first portion

of the distal convoluted tubules. When the urine is neutral the cells above the descending limb of the loop of Henle are alkaline to erythrolitmin, and those below this point appear acid.

The granules within the endothelial cells of the glomerulus and the epithelial cells of the first portion of the proximal convoluted tubules are always alkaline to erythrolitmin; while those within the cells of the final portion of the distal convoluted tubules are always acid regardless of the reaction of the urine. Only after complete suppression of urine as result of massive doses of acid do granules of the sort first mentioned manifest the color indicative of acidity.

The interpretation of the findings waits upon further work.

BIBLIOGRAPHY.

1. Heidenhain, R., in Hermann L., Handbuch der Physiologie, Leipsic, 1883, v, 279.
2. Nussbaum, M., *Arch. ges. Physiol.*, 1878, xvi, 139.
3. Nussbaum, M., *Arch. ges. Physiol.*, 1878, xvii, 580.
4. von Sobieranski, W., *Arch. exp. Path. u. Pharmakol.*, 1895, xxxv, 144.
5. Suzuki, T., Zur Morphologie der Nierensekretion unter physiologischen und pathologischen Bedingungen, Jena, 1912.
6. Shafer, G. D., *Am. J. Physiol.*, 1908, xxii, 335.
7. Stieglitz, E. J., *Arch. Int. Med.*, 1924, xxxiii, 483.
8. Rous, P., *J. Exp. Med.*, 1925, xli, 379.
9. Rous, P., *J. Exp. Med.*, 1925, xli, 399.
10. Rous, P., *J. Exp. Med.*, 1925, xli, 451.
11. Rous, P., *J. Exp. Med.*, 1925, xli, 739.
12. Cushny, A. R., The secretion of urine, London, 1917.
13. Marshall, E. K., Jr., and Crane, M. M., *Am. J. Physiol.*, 1924, lxx, 465.
14. Wearn, J. T., and Richards, A. N., *Am. J. Physiol.*, 1924–25, lxxi, 209.
15. Richards, A. N., and Wearn, J. T., *J. Am. Med. Sc.*, 1923, lxxx, 1644.
16. Wearn, J. T., *Am. J. Physiol.*, 1922, lix, 490.
17. Bieter, R. N., and Hirschfelder, A. D., *Am. J. Physiol.*, 1924, lxviii, 326.
18. Marshall, E. K., Jr., and Vickers, J. L., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 1.
19. Dreser, H., *Z. Biol.*, 1885, xxi, 41.
20. Dreser, H., *Z. Biol.*, 1886, xxii, 56.
21. Grützner, P., *Arch. ges. Physiol.*, 1882, xxiv, 461.
22. Rous, P., *Science*, 1924, lx, 363.
23. Edwards, J. G., and Marshall, E. K., Jr., *Am. J. Physiol.*, 1924, lxx, 489.
24. Elman, R., Drury, D. R., and McMaster, P. D., *J. Exp. Med.*, 1928, xlvii, 777.

25. Scheitz, P., *Z. anal. Chem.*, 1910, xlix, 735.
26. Scheitz, P., *Z. anal. Chem.*, 1910, xlix, 736.
27. Thorpe, E., A dictionary of applied chemistry, London, 3rd edition, 1922, iv.
28. Haldane, J. B. S., Hill, R., and Luck, J. M., *J. Physiol.*, 1923, lvii, 301.
29. Gamble, J. L., Ross, G. S., and Tisdall, F. F., *Am. J. Dis. Child.*, 1923, xxv, 455.
30. Addis, T., MacKay, E. M., and MacKay, L. L., *J. Biol. Chem.*, 1926, lxxi, 157.
31. Carrel, A., and Guthrie, C. C., *Science*, 1906, xxiii, 394.

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ETIOLOGY OF OROYA FEVER.

XII. INFLUENCE OF MALARIAL INFECTION (PLASMODIUM INUI?), SPLENECTOMY, OR BOTH, UPON EXPERIMENTAL CARRION'S DISEASE IN MONKEYS.

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Mixed infections with *Bartonella bacilliformis* and plasmodia are not uncommon in regions where both malaria and Carrion's disease (verruga peruana, Oroya fever) are endemic. Both parasites induce swelling of the spleen, and both invade the red blood cells. It is conceivable that the preexistence of malaria might have an unfavorable influence upon a subsequent infection with *Bartonella bacilliformis*, as a result of impairment of the defensive powers of spleen and blood by the plasmodia, or that the introduction of plasmodia during the course of a dormant or partially controlled verruga infection might result in renewed invasion of the blood and lymphatic system by *Bartonella bacilliformis*. The abrupt development of malignant Oroya fever in the course of apparently benign verruga would be explicable on this latter hypothesis.

The opportunity to obtain definite experimental data on the effect of malaria infection on the course of experimental verruga presented itself when malaria parasites were found in the blood of a monkey (*Macacus cynomolgus*) which had been splenectomized in connection with an experiment to determine what influence the operation would have upon the susceptibility of monkeys to *Bartonella bacilliformis*.

Protocol 1.—Macacus cynomolgus 1-M, splenectomized on Dec. 1, 1926, to serve as control for *M. cynomolgos 2-S*, which was splenectomized the same day and inoculated with *Bartonella bacilliformis*. Examination of the blood of Monkey 1-M was made daily in order that the appearance of any *Bartonella*-like intracor-

puscular elements (such as appear in splenectomized rats[1]) might be detected. These elements were not found, but the examinations revealed plasmodia[2] on Dec. 6, 9, 10, 11, and 16, 1926, and the parasites were still present at the time of death on Mar. 10, 1927. No fever was detected in this animal at any time. The monkey had shown no signs of illness during several months of captivity previous to the operation. It would appear that it was a chronic malarial parasite carrier, a condition already established for man. Death may have been due to aggravation of the malarial infection through splenectomy.

Preexisting or Concomitant Malarial Infection and Verruga.

A *Macacus rhesus* intravenously inoculated with the blood of *Macacus cynomolgus 1-M* on December 10 soon developed fever, and the malarial parasites were found in the blood. One month later verruga material was intradermally inoculated. In due course the cutaneous lesions appeared and attained moderate size. Blood taken on February 3, 1927, yielded cultures of *Bartonella bacilliformis*, but no severe form of blood invasion and anemia developed. The animal recovered from both infections within a few weeks.

Protocol 2.—Macacus rhesus 2-M, inoculated intravenously with 0.5 cc. of the blood of Monkey 1-M on the day when plasmodia were first noticed (Dec. 10, 1926). The febrile reaction began on Dec. 15, that is, 5 days after inoculation, and continued daily for 9 successive days, being high (105–106.2°F.) for 5 days and moderate (104.2–104.6°) for 4 days longer. The plasmodia were present on Dec. 16. The temperature was normal (about 102°) from Dec. 26 to Jan. 3 but rose to 104.6° on Jan. 4, 1927, and to 106° on Jan. 5. Plasmodia were found in the blood on Jan. 3. After Jan. 5 there was no more fever. On Jan. 10, 1927, cultures of *Bartonella bacilliformis* and a saline suspension of the nodule of *M. rhesus 73* were intradermally inoculated. The signs of verruga were already evident on Jan. 19, and by Feb. 3 the nodules were of moderate size, and the blood had a culture titer of 1:100. The animal had no further paroxysm of malaria and recovered from both infections within a few weeks.

Another monkey was inoculated simultaneously with malaria and verruga material.

Protocol 3.—Macacus rhesus 3-M was inoculated on the same day as *M. rhesus 2-M* with 0.5 cc. of blood containing plasmodia and in addition received, both

[1] Mayer, M., *Arch. Schiffs.- u. Tropenhyg.*, 1921, xxv, 150. Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Woch.*, 1926, v, 559; *Arch. Schiffs.- u. Tropenhyg.*, 1927, xxxi, 295. Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 235.

[2] The parasite would appear to be *Plasmodium inui* (Wenyon, C. M., Protozoology, New York, 1926, ii, 968–973).

intravenously and intradermally, cultures of *Bartonella bacilliformis* and a suspension of verruga tissue. The animal's blood showed plasmodia on Dec. 16, 1926, 3 days before the onset of fever. The first paroxysm lasted 3 days (temperature 104.6°, 106.2°, 106.2°). The second rise of temperature (to 105.4°) was first detected on Dec. 27, temperatures not being taken daily during the Christmas holidays, and fever was still present on Dec. 28 (104°). Plasmodia were present in the blood on Jan. 3, and there was fever on Jan. 4, 5, and 6 (104°, 105.8°, 104°). Verruga nodules arose at the sites of intravenous inoculation into the saphenous veins (where several unsuccessful punctures had been made in the course of intravenous injection), but none at the sites of intradermal injections on the abdomen. Blood cultures made on Dec. 21, 1926, failed to yield cultures of *Bartonella bacilliformis*. The temperature of 106.2°F., recorded on that day, may have been due either to malaria or to verruga. A second injection of virulent verruga material intradermally on Jan. 10, 1927, resulted in only slight induration at the sites of inoculation, but the nodules on the back of the legs reached moderate size, and marked edema of the scrotum developed. The animal recovered.

The experiments outlined gave no evidence that the malarial infection in *Macacus rhesus* influenced unfavorably the result of infection with *Bartonella bacilliformis* when the plasmodia were introduced a month previous to inoculation with verruga or when both parasites were simultaneously inoculated.

Malarial Infection during Convalescence from Verruga.

The lesions resulting from experimental infection with *Bartonella bacilliformis* in monkeys usually heal within a few months, the microorganisms persisting longest in the spleen and lymph glands, where they can be demonstrated late in convalescence. It was of interest to determine whether, at this stage, the infection would again become active as a result of introduction of plasmodia.

As the protocols show, the inoculation of malaria parasites during convalescence had no influence whatever upon the course of recovery. In one instance the animal was reinoculated with virulent verruga material a month after the introduction of the plasmodia, but reinfection was not induced. The malarial infection apparently had not interfered with the development of immunity.

Protocol 4.—Macacus rhesus 4-M had had an infection with *Bartonella bacilliformis* during the autumn, but the skin lesions had almost healed. It was inoculated with 0.5 cc. of the malarial blood intravenously on Dec. 10, 1926. There

was fever lasting for 6 days, Dec. 22 to 27, 1926 (temperatures varying from 104.4° to 105°), and the blood was positive for plasmodia on Jan. 3, 1927. Blood culture was negative for *Bartonella bacilliformis* on Dec. 21, 1926, and the skin lesions had disappeared.

Macacus rhesus 5-M, also recovering from verruga, was inoculated with 0.5 cc. of malarial blood at the same time as the foregoing animal. Fever began on Dec. 16, 1926, and lasted 5 days (temperatures 104°–106.8°). Plasmodia were present on Dec. 16, 1926, and on Jan. 3, 1927. On Dec. 28, 1926, the temperature rose to 104.4° but fell to 103.6° the next day, and there were no later paroxysms. On Jan. 8, 1927, verruga material was inoculated intradermally. No skin lesions appeared at the old sites, and none developed at the sites of the new inoculations, and the blood remained negative for *Bartonella bacilliformis* by culture. Observation ended Jan. 29, 1927.

Effect of Splenectomy upon Verruga Peruana.

In connection with the problem of the nature of the intracorpuscular bodies known as *Bartonella muris*,[1] which appear in the red cells of rats following splenectomy, it became of interest to determine what effect splenectomy would have upon the course of experimental infection with *Bartonella bacilliformis*.

A monkey splenectomized on November 12, 1926, and inoculated soon afterwards with verruga material, developed the severe type of Carrion's disease (Oroya fever), with marked anemia, extensive edema, emaciation, and skin lesions. Even more violent reactions than the one in this animal (*Macacus rhesus 1-S*) are occasionally observed in *rhesus* monkeys as a result of inoculation with *Bartonella bacilliformis*,[3,4] and hence the severity of the infection in this instance cannot be ascribed to the effect of splenectomy. Moreover, *M. cynomolgus 2-S*, which was subjected to splenectomy and simultaneously inoculated with nodular material from Monkey 1-S, showed a moderate reaction only. Although the blood titer was high (1:100,000), there were no other evidences of systemic infection, and the nodules were of medium size. The control monkey in this instance (*M. cynomolgus 5-T*) reacted more severely than the splenectomized animal, although the blood titer was only 1:10, as was that of a *rhesus* control.

[3] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

[4] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

Protocol 5.—Macacus rhesus 1-S, splenectomized Nov. 12, 1926, was inoculated 3 days later intravenously with 1 cc. of culture of *Bartonella bacilliformis* and 1 cc. of saline suspension of nodular tissue from *Macacus rhesus 2-T*.[5] The same material was injected intradermally. A temperature of 104° to 104.6°F. was recorded on Nov. 11, 24, Dec. 10, 20, and 21, and on Jan. 15, 1927. Except on these 6 days the animal was afebrile. Blood cultures were positive on Nov. 24 (1:10,000), Dec. 8 (1:100,000), and Dec. 28 (1:100). The organisms were sufficiently numerous in the blood on Dec. 3 to be detected by examination of stained films. The nodules developed to moderately large size and persisted for about 2 months. From Dec. 11, 1926, to Jan. 3, 1927, there was general edema, especially marked in the scrotum, and a generalized nodular eruption.

Blood Counts.

	R.B.C.	Hemoglobin (Sahli) *per cent*
Nov. 12	5,456,000	90
Nov. 24	4,424,000	80
Dec. 27	3,080,000	50

The animal had completely recovered by Feb. 7, 1927.

M. cynomolgus 2-S, splenectomized Dec. 1, 1926, and inoculated intradermally with a culture of *Bartonella bacilliformis* and a saline suspension of nodular tissue from *M. rhesus 1-S*. Nodules of moderate size had developed by Jan. 14, 1927. Blood taken on Jan. 6, 1927, yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100,000. The nodules had disappeared by Feb. 9, 1927.

M. cynomolgus 5-T[5] was inoculated in the same way and with the same material as the foregoing animal but was not splenectomized. There was no fever at any time, but enormous cherry-red nodules developed on the eyebrows and abdominal wall, reaching their maximum growth on Dec. 27, 1926. The blood titer was 1:10 on Jan. 6, 1927, but blood culture was negative on Jan. 24.

Splenectomy did not induce relapse of experimental verruga infection in two animals which had recovered (*M. rhesus 40* and *41*[3]), nor did removal of the spleen render recovered animals susceptible to reinoculation with *Bartonella bacilliformis* (*M. rhesus 49, 50, 68*). Observation continued for a period of 1 month after inoculation of *Bartonella bacilliformis* into these animals failed to reveal any skin lesions, and blood cultures were uniformly negative throughout this period.

Effect of Splenectomy and Malarial Infection Together upon Verruga Peruana.

Two animals were subjected to splenectomy as well as to infection with plasmodia and *Bartonella bacilliformis*. One succumbed to the

[5] Noguchi, H., *J. Exp. Med.*, in press.

malarial infection before the verruga lesions had had time to develop; the other had a moderately severe infection with *Bartonella bacilliformis* and while recovering died of tuberculosis.

Protocol 6.—Macacus rhesus 6-M was splenectomized and at the same time inoculated with 0.5 cc. of malarial blood on Dec. 10, 1926. The plasmodia were found in the blood on Dec. 16, and 4 days later the temperature rose and remained high for 3 successive days (104.2–106°). The second paroxysm began on Dec. 29, 1926, and lasted for 8 days (temperatures 104–106.6°). The plasmodia were very numerous on Jan. 3. The animal was inoculated with verruga material on Jan. 8, 1927, but succumbed to the malarial infection on Jan. 14, 1927, before the verruga lesions had had time to develop. *Bartonella bacilliformis* was not recovered in blood cultures.

Macacus rhesus 7-M was splenectomized on Dec. 10, 1926, and at the same time inoculated with 0.5 cc. of malarial blood and 0.5 cc. of verruga cultures and tissue, the latter intradermally as well as intravenously. The first paroxysm occurred on Dec. 20 and lasted 4 days (temperatures 104.2–106°), the second, beginning Jan. 3, lasted 5 days (temperatures 104.6–106.4°). Examinations for plasmodia were positive on Dec. 16, 1926 (+), and on Jan. 3, 1927 (+++). Blood taken on Dec. 21, 1926, yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000. The animal died of tuberculosis on Jan. 10, 1927.

SUMMARY.

The experiments reported were designed to determine the influence of malarial infection (*Plasmodium inui?*), splenectomy, or both combined, upon the course and character of experimental infection with *Bartonella bacilliformis* in monkeys (*Macacus rhesus* and *M. cynomolgus*).

Blood withdrawn from a monkey showing spontaneous malarial infection was inoculated intravenously into monkeys (*a*) 1 month prior to inoculation with virulent verruga material, (*b*) simultaneously with the verruga material, and (*c*) during convalescence from verruga infection of moderate severity. All the monkeys contracted the malarial infection and suffered one to three paroxysms during a period of about a month. The verruga lesions appeared in the inoculated animals in due course, were of average size, remained for the usual length of time, and *Bartonella bacilliformis* was recovered in culture from blood which also contained the plasmodia. The lesions in the convalescent animals continued to heal at the normal rate, and blood cultures were negative for *Bartonella bacilliformis*, as is usual during

convalescence. One of the recovering animals was reinoculated with virulent verruga material a month after the injection of the malarial blood, but neither did new lesions arise nor old ones recur. The malarial infection, therefore, had no effect upon the course of verruga or upon the establishment of immunity to *Bartonella bacilliformis*, hence it would appear that malaria and verruga may coexist in the same individual without unfavorable effect of one disease upon the course of the other.

Similarly, splenectomy led to no appreciable aggravation of *Bartonella* infection. One monkey subjected to splenectomy and inoculated with verruga material shortly afterwards had an unusually severe reaction, but another, which was infected with material from the first and simultaneously splenectomized, reacted only moderately, while the non-splenectomized control showed a severer type of cutaneous infection. Even the combination of splenectomy and malarial infection did not appreciably aggravate the experimental verruga. Neither relapse of verruga nor reinfection with *Bartonella bacilliformis* was induced in convalescent or recovered monkeys as a result of splenectomy.

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THE AMMONIACAL SILVER SOLUTIONS USED IN NEUROPATHOLOGY.

THEIR STAINING PROPERTIES, CHEMISTRY AND METHODS OF PREPARATION.

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The methods of staining with silver salts, developed so largely by Fajersztajn and Bielschowsky in Germany, and by Ramón y Cajal and Del Rio-Hortega in Spain, are receiving increasing attention. Several procedures have been described for preparing the ammoniacal silver solutions which are used in these methods, and selective staining results have been attributed to each. The conflicting claims have led at times to actual controversy, and the present investigation aims to clarify this subject.[1]

(1) The method of Fajersztajn,[2] described in 1901, is of both historical and theoretical interest (as will be shown later). To a weak solution (exact concentration not given) of silver nitrate ($AgNO_3$), Fajersztajn added ammonia until the brown precipitate which formed at first had completely redissolved. He then added more silver nitrate until a precipitate began to form again; the solution, supposedly "freed of excess ammonia" in this way, was filtered through hardened "analytical" filter paper.

(2) In 1902, Bielschowsky introduced his classic method for the silver staining of the axis-cylinder.[3] His method of preparing the silver solution has undergone slight alterations at various times; as learned from him personally during the fall of 1926, it may be outlined as follows:

To 5 cc. of 10 per cent silver nitrate are added 8 drops of a 40 per cent solution of sodium hydroxide in a glass-stoppered graduate. This is shaken well. Then a

1. It is a privilege to express here our gratitude to Professors Bielschowsky, Ramón y Cajal, and Del Rio-Hortega for generous help and instruction in the details of these methods, during the spring, summer and fall of 1926 (L. S. K.).

2. Fajersztajn, J.: Ein neues Silberimprägnationsverfahren als Mittel zur Färbung der Axenzylinder, Neurol. Centralbl. **20**: 98, 1901.

3. Bielschowsky, M.: Die Silberimprägnation der Axenzylinder, Neurol. Centralbl. **21**: 579, 1902.

solution of 25 per cent ammonia (specific gravity about 0.9) is added rapidly, drop by drop, with almost constant shaking. The addition of ammonia is stopped when the large brown granules suddenly disappear, leaving a muddy brown solution which is barely translucent but in which individual granules are not clearly visible. Occasionally one more drop of ammonia may be added after this point is reached, but usually the resolution of the silver is ended here. This solution is diluted to 25 cc. with distilled water, and before using is filtered through ordinary filter paper which has been rinsed with distilled water.

(3) The essential difference between the method of Ramón y Cajal and the foregoing method is the thorough washing of the precipitate which Cajal recommends.[4] To 10 cc. of a solution of 10 per cent silver nitrate are added 12 drops of 40 per cent sodium hydroxide in a graduated cylinder. Distilled water is added to the precipitate, and after vigorous shaking the precipitate is allowed to settle again and the supernatant water is decanted. This is repeated at least five times, after which distilled water is added to make a total volume of 60 or 70 cc. Concentrated ammonia is then added, drop by drop, with frequent shaking, until the precipitate is almost, but not completely, dissolved. The undissolved residue is allowed to remain in order to avoid as far as possible an "excess" of ammonia.

(4) Del Rio-Hortega[5] introduced the use of sodium or lithium carbonate in place of sodium hydroxide or ammonia for the initial precipitation of the silver. To 5 cc. of 10 per cent silver nitrate he adds from 15 to 20 cc. of 5 per cent sodium carbonate (Na_2CO_3), or of a mixure of sodium and potassium carbonate, or of a saturated solution of lithium carbonate. Del Rio-Hortega believes that the yellow-white precipitate which forms tends to darken quickly in the light, necessitating rapid solution with ammonia. For this reason, he quickly adds strong ammonia, caring less about the presence of an excess than do the other workers. The resulting solution may at times be used without dilution; at others, with dilution to 30 or 40 cc., or to 75 cc.

The chemistry of such solutions has been the subject of many physicochemical investigations, which indicate that in all of these ammoniacal solutions of silver compounds, the silver exists largely in the form of the complex silver-ammonia-cation $[Ag(NH_3)_2^+]$. (Full references are given in an article by Mellor.[6]) In other words, the silver ion is the same whichever of these methods is used in pre-

4. Ramón y Cajal, S.: Elementos de histologia normal y de tecnica micrografica, ed. 8, Madrid, 1926, p. 729.

5. Rio-Hortega, P. Del: Noticia de un nuevo y fácil método para la coloración de la neuroglia y del tejido conjuntivo, Trav. del lab. de inv. biol. **15**: 378, 1918.

6. Mellor, J. W.: A Comprehensive Treatise on Inorganic and Theoretical Chemistry, London, Longmans, Green & Co., 1923, vol. 3, p. 382.

paring it; but the activity of the ion will be found to show striking variations, depending on the other constituents of the solution.

These methods of preparation may be grouped under three headings. When ammonia alone is used both for precipitation and solution, the final solution may best be spoken of as "ammoniacal silver nitrate;" the chief product of the reaction is silver diammino-nitrate [$Ag(NH_3)_2NO_3$]. When the precipitate is produced with sodium hydroxide, and ammonia is used only to redissolve, one may speak of the solution as "ammoniacal silver hydroxide;" the chief constituent is the strongly dissociated base, silver diammino-hydroxide [$Ag(NH_3)_2OH$]. With sodium carbonate as precipitant, followed by ammonia, the solution may be called "ammoniacal silver carbonate," with silver diammino-carbonate [$(Ag(NH_3)_2)_2CO_3$] as its main component.

It will be shown that the amount of ammonia required to prepare these solutions varies with, and is actually a measure of, their alkalinity; and that the variations in staining activity correlate with this.

AMOUNT OF AMMONIA NECESSARY FOR COMPLETE SOLUTION OF THE SILVER COMPOUND, AND THE EFFECT OF DIFFERENT HYDROXYL ION CONCENTRATIONS ON THIS.

Ammonia Used Alone (*"Ammoniacal Silver Nitrate"*).—From the formula of the complex silver cation [$Ag(NH_3)_2^+$], it is evident that two molecules of ammonia are required for combination with each atom of silver. It was found, however, that 10 cc. of 1.2 molar ammonia solution, when added to 10 cc. of 0.6 molar silver nitrate[7] (i. e., a ratio of two molecules of ammonia to one atom of silver) left an undissolved residue of brown precipitate, which went into solution only on the addition of 0.3 cc. more of the ammonia. This yields the ratio 2.06:1, for the ratio of molecules of ammonia to molecules of

7. In the present investigation, a 0.6 molar solution of silver nitrate (10.2 per cent) has been used, because it closely approximates the 10 per cent solution customarily used in histologic laboratories. An ammonia solution of 1.2 molar strength has also been used, so that equal volumes of these two solutions were chemically equivalent. The preparation of these solutions is described later.

silver nitrate, which agrees closely with the results of previous investigators:

	Mean
Prescott: Chem. News **42**: 31, 1880	2.06
Reychler: Ber. d. deutsch. chem. gesellsch. **16**: 990, 1883	2.03
Draper: Pharm. J. **17**: 487, 1886	2.06
Hertz: Z. anorg. Chem. **67**: 248, 1910	2.11
	2.065

The explanation of the excess ammonia is found in the following considerations: (a) Silver nitrate reacts directly with ammonia molecules in solution, to give silver diammino-nitrate, $AgNO_3 + 2NH_3 = Ag(NH_3)_2NO_3$. (b) This reaction, however, stops short of completion, because the complex ion, $Ag(NH_3)_2^+$, although stable, dissociates to a small extent to yield significant concentrations of silver ion (Ag^+) and of ammonia free in solution. (c) Ammonia in solution exists in three forms: as molecules of ammonia, as hydrated ammonia ($NH_3 + H_2O = NH_4OH$), and as the dissociated ions of NH_4OH, NH_4^+ and OH^-.[8] As a result, there occur in the solution concentrations of both silver and hydroxyl ions which are sufficient to cause a precipitate of the slightly soluble substance, silver hydroxide. This situation is represented in formula 1.

Formula 1

$$Ag(NH_3)_2NO_3 \rightleftarrows NO_3^- + Ag(NH_3)_2^+ \rightleftarrows 2NH_3 + Ag^+$$

$$+ \quad + \quad +$$

$$H_2O$$

$$\downarrow\uparrow$$

$$NH_4OH \rightleftarrows OH^- + NH_4^+$$

$$\downarrow\uparrow$$

$$AgOH$$

(dissolved)

$$\downarrow\uparrow$$

$$AgOH \rightleftarrows \tfrac{1}{2}(Ag_2O + H_2O)$$

(solid)

(d) Since, in general, the dissociation of a substance is inhibited by the presence of one of the products of dissociation, the addition of an

8. It is probable that ammonium hydroxide is itself a strong base, and therefore largely dissociated, but that only a small amount of ammonium hydroxide forms so that the total behavior of an ammonia solution is that of a weak base.

excess of ammonia will depress the dissociation of $Ag(NH_3)_2^+$, and will therefore decrease the concentration of silver ion arising from it. And since, moreover, the addition of excess ammonia causes a proportionally greater decrease in the concentration of silver ion than the concomitant increase in hydroxyl ion, the solution of the silver hydroxide (or oxide) precipitate is made possible when a sufficient amount of ammonia has been added. In the present case, as has been said, this occurs with an excess of 0.3 cc. of 1.2 molar ammonia.

To clarify the subsequent discussion of the effects of adding sodium hydroxide to this reaction, it is necessary to explain the principles of precipitation and solution a little further. For the precipitation of a substance from solution, it is necessary that the ions of this substance be present in quantities such that when the two concentrations are multiplied together, the product is greater than a certain figure, which is called the "solubility product—S" of the substance. This solubility product is an experimentally determined value which is characteristic for any compound. It is the product of the concentrations of the ions in a pure saturated solution of the compound.

It should be clear, therefore, that, after dissolving a compound in water to the limit of its solubility, if anything occurs to increase the concentration of one of its ions, in order to hold the compound in solution there must be produced an exactly proportionate decrease in concentration of the other ion, since the product of these two concentrations cannot exceed this constant value if the substance is to remain in solution. It will be seen, in the next section, that the use of sodium hydroxide, by raising the concentration of hydroxyl ions, necessitates the use of a much larger excess of ammonia in order to secure a sufficient decrease in the concentration of silver ions to make possible complete solution of the precipitated silver oxide.

With Sodium Hydroxide and Ammonia ("Ammoniacal Silver Hydroxide").[9]—From the foregoing discussion it should be evident that a clear solution of ammoniacal silver nitrate inevitably contains an excess of ammonia, besides definite concentrations of silver and hydroxyl ions. If to such a solution there is added an equivalent amount of sodium hydroxide (i. e., in this case, 10 cc. of 0.6 molar), the strong base markedly increases the hydroxyl ion concentration.

9. In discussing these solutions, it is preferable to speak of the hydroxide, rather than of the oxide, because when they are in solution both the silver oxide and the ammoniacal silver oxide, like the oxides of all strong bases, largely take the form of the hydroxide; and it is in solution as the hydroxide that all of their reactions occur.

The product of the concentrations of silver ion and hydroxyl ion becomes much greater than the solubility product of silver hydroxide, and as a result there is a precipitation of the major portion of the silver present in the solution. The precipitated oxide, nevertheless, may be returned to solution by adding a still greater excess of ammonia. This ammonia further represses the dissociation of the complex ion, $Ag(NH_3)_2^+$, and thereby reduces the silver ion concentration until the product of the two ions is no longer greater than the solubility product of silver hydroxide, whereupon a return to solution becomes possible. Actually, 5.5 cc. more of 1.2 molar ammonia were required (table 1). This corresponds to a value of 3.34 for the ratio NH_3:-

TABLE 1.

Cubic Centimeters of 1.2 Molar Ammonia Necessary to Secure Complete Solution.

(1)	10 cc. of 0.6 M $AgNO_3$	10.3
(2)	10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.6 M NaOH	16.8
(3)	10 cc. of 0.6 M $AgNO_3$ plus 15 cc. of 1.2 M NH_3 plus 10 cc. of 0.6 M NaOH (i. e. reverse order)	1.7 = total of 16.7

$AgNO_3$, which is in good agreement with the results of former investigations:

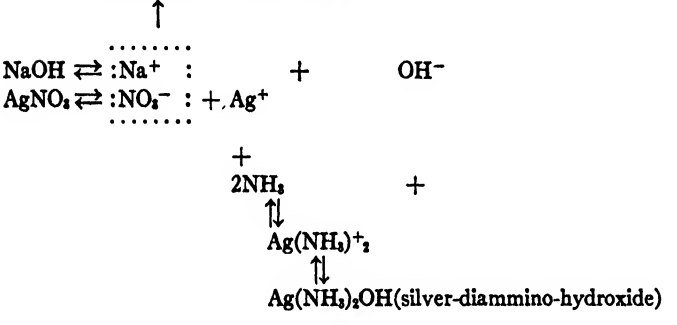
	Mean
Whitney and Melcher: J. Am. Chem. Soc. **25**: 70, 1903	3.28
Euler: Ber. d. deutsch. chem. gesellsch. **36**: 1854, 1903	3.03
Olmer: Bull. Soc. chim. **35**: 333, 1924	3.32
	3.210

In the present case, the effect of the added ammonia is almost exclusively on the silver ion concentration and not on the hydroxyl ion concentration, since the presence of the strong base depresses the dissociation of the ammonium hydroxide and renders its contribution to the final hydroxyl concentration insignificant. Furthermore, the same amount of ammonia is required and the final solution is the same if the procedure is modified by first adding the alkali to silver nitrate

and then treating with ammonia, which is the more usual procedure in histologic laboratories.

The strong alkalinity of a solution of equivalent amounts of silver nitrate and sodium hydroxide in ammonia is not dependent on the presence of an excess of sodium hydroxide over that which is exactly equivalent to the initial amount of silver nitrate. It is due to the fact that silver-diammino-hydroxide, which is formed in the reaction, is almost as strong a base as sodium hydroxide itself. The situation is represented in formula 2.

Formula 2



It is evident from formula 2 that if no excess of sodium hydroxide has been used, washing away the soluble sodium nitrate before adding the ammonia cannot appreciably affect the hydroxyl ion concentration of the final solution, since sodium hydroxide and silver-diammino-hydroxide are of comparable strength as bases. If, on the other hand, less than one equivalent of sodium hydroxide is used, there will be a lower hydroxyl concentration, and less ammonia will be needed. With an excess of sodium hydroxide the reverse will be true (table 2). In the latter case, moreover, washing the precipitate will actually wash away a certain amount of the excess sodium hydroxide, and so diminish the amount of ammonia required for solution after the washing. This is seen, in table 3, to be the essential difference between the Bielschowsky and the Cajal methods of preparing the solutions, in both of which a large excess of sodium hydroxide is used, but in the latter of which the precipitate is washed five or six times while in the former it is not washed at all. It is also evident from table 3 that

complete removal of the excess base is not achieved even by ten washings of the precipitate, as there is still necessary a much larger amount

TABLE 2.

Cubic Centimeters of 1.2 Molar Ammonia Necessary to Secure Complete Solution of the Precipitate.

(1) 10 cc. of 0.6 M $AgNO_3$	10.3
(2) 10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.6 M NaOH with no washing of the precipitate	16.8
(3) 10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.6 M NaOH with 8 washings of the precipitate	16.7
(4) 10 cc. of 0.6 M $AgNO_3$ plus 5 cc. of 0.6 M NaOH	15.2–15.6
(5) 10 cc. of 0.6 M $AgNO_3$ plus 20 cc. of 0.6 M NaOH	21.1

TABLE 3.

*Cubic Centimeter of 1.2 Molar Ammonia Necessary to Secure Complete Solution.**

(1) Bielschowsky Method 10 cc. of 10 per cent $AgNO_3$ plus 12 drops of 40 per cent NaOH with no washing of the precipitate	22
(2) Cajal Method 10 cc. of 10 per cent $AgNO_3$ plus 12 drops of 40 per cent NaOH with 10 washings of the precipitate	19.5

* If concentrated ammonia is used, these differences are too small to be demonstrated, representing less than one drop of the solution usually used in these methods.

of ammonia than is needed for the same amount of silver when only one equivalent of sodium hydroxide is used in the first place.

The actual hydroxyl ion concentrations of these solutions can be calculated from the amount of ammonia required to prepare them.

These calculations are presented in the full discussion of these solutions which will shortly be published by one of us (D. D.).

With Sodium Carbonate and Ammonia ("Ammoniacal Silver Carbonate").—Before considering the nature of the ammoniacal silver carbonate or of ammoniacal silver nitrate plus sodium carbonate, the foregoing discussion may first be summarized as follows:

In ammoniacal silver nitrate solution there are present, as a result of dissociation of the complex ion, definite and significant concentrations of silver ion, ammonia, and hydroxyl ion; the product of silver and hydroxyl ion concentrations, however, never exceeding the solubility product of silver hydroxide. If either the silver or the hydroxyl ion concentration is increased, by adding silver nitrate on the one hand or sodium hydroxide on the other, then silver hydroxide will be precipitated. In both cases, subsequent addition of a definite excess of ammonia will redissolve the precipitate.

If, however, a substance is added to ammoniacal silver nitrate which increases neither the silver nor the hydroxyl ion concentrations, and the ions of which form with silver ion no salt which is less soluble than silver hydroxide, then no precipitate will be formed and no further excess of ammonia will be required. Sodium carbonate is of this nature, since its hydroxyl concentration is less than that already existing in the ammoniacal solution, and since the silver carbonate which might be formed by combination of its acidic ion (CO_3—) is more soluble than silver hydroxide. Hence the addition of sodium carbonate to ammoniacal silver nitrate does not produce precipitation; or, in other words, the precipitate produced by adding sodium carbonate to silver nitrate solution requires no more ammonia for resolution than does silver nitrate itself. Washing the silver carbonate precipitate before adding ammonia does not have any effect on the final solution obtained, since here, too, the soluble sodium nitrate which would otherwise be present is of no consequence.

Although the actual concentration of hydroxyl ions is not altered, it is obvious that the addition of carbonate to the solution of silver in ammonia provides the final solution with a large amount of buffer. As a result, its titratable alkali is relatively high; that is, it will require a much larger amount of acid to change its hydroxyl ion concentration than that of a solution of ammoniacal silver nitrate.

A rough comparison of the titratable alkalinity of solutions of silver nitrate in ammonia alone, in sodium hydroxide and ammonia and in sodium carbonate (Na_2CO_3) and ammonia is presented in table 5. With thymol blue as indicator, an arbitrary end point at about pH 8.4 to 8.6 was taken; and to 10 cc. of the solution tested, 0.12 molar

TABLE 4.

Cubic Centimeters of 1.2 Molar Ammonia Necessary to Secure Complete Solution of the Precipitate.

(1)	10 cc. of 0.6 M $AgNO_3$	10.3
(2)	10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.3 M Na_2CO_3 with no washings of the precipitate	10.4
(3)	10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.3 M Na_2CO_3 with 6 washings of the precipitate	9.8*
(4)	10 cc. of 0.6 M $AgNO_3$ plus 20 cc. of 0.3 M Na_2CO_3	10.4
(5)	Hortega Silver Method 10 cc. of 10 per cent $AgNO_3$ plus 30–40 cc. of 5 per cent Na_2CO_3	10.4

* This diminution of the amount of ammonia needed is due to the fact that the silver carbonate is soluble, so that every time the precipitate is washed a perceptible amount of the silver salt goes into solution and is lost. That this is true is shown by the fact that each decanted washing fluid gives a positive test for silver even when centrifugalized to rid it of finely divided particles of the precipitate itself. The silver lost in this way lessened, of course, the amount of ammonia needed for dissolving the precipitate which remained. This is true of silver hydroxide as well; but to a much slighter extent because silver hydroxide is much less soluble in water than is silver carbonate.

nitric acid was added until the alkalinity of the solution was reduced to this point. At this pH the color of the thymol blue indicator changes from mauve to yellow. While not absolutely sharp, the end point is clearly recognizable.

It will be seen later that the high buffer content of the ammoniacal silver carbonate solution affects its activity in the same direction as

does the high hydroxyl ion content of the ammoniacal silver solution which is made with sodium hydroxide, but to a less extreme degree.

We may summarize, then, certain essential facts about the chemistry of these solutions, as follows:

TABLE 5.

Titration of Relative Amounts of Reserve Alkalinity of Ammoniacal Silver Solutions, with 0.12 Molar Nitric Acid.

(Silver solution, 10 cc.; thymol blue, 4 drops; titrated to pH 8.4–8.6.*)

	Cc. of 0.12 M HNO_3
(1) 10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.6 M NaOH (precipitate not washed) NH_3 to complete solution (16.8 cc. of 1.2 M) Diluted to 50 cc. total volume	20.5
(2) The same, except that the precipitate is washed before adding ammonia	20
(3) 10 cc. of 0.6 M $AgNO_3$ plus 10 cc. of 0.3 M Na_2CO_3 (precipitate not washed) NH_3 to complete solution (10.4 cc. of 1.2 M) Diluted to 50 cc. total volume	7.1*
(4) The same, except that the precipitate is washed before adding the NH_3	6.9*
(5) 10 cc. of 0.6 M $AgNO_3$ plus NH_3 to complete solution (10.3 cc. of 1.2 M) Diluted to 50 cc. total volume	2

* Despite the formation of a cloudy white precipitate in the carbonate solutions, these end points are approximately correct.

1. To dissolve silver nitrate, more ammonia is needed than that which is calculated from the structure of the ammoniacal silver cation which is formed.

2. If silver nitrate plus an exact chemical equivalent of sodium hydroxide is used, still more ammonia is required. This is due to the high hydroxyl ion concentration of the resulting solution; which, in turn, depends not on the presence of excess sodium hydroxide but on

the fact that the silver diammino-hydroxide is itself a strong base.

3. The addition of one chemical equivalent of sodium carbonate to silver nitrate does not, on the other hand, make necessary the use of any more ammonia than with the silver nitrate alone. It is evident, therefore, that solutions of ammoniacal silver nitrate and ammoniacal silver carbonate have approximately identical hydroxyl ion concentrations— and that this hydroxyl ion concentration is much less than that of a solution of ammoniacal silver hydroxide.

4. Between solutions of ammoniacal silver nitrate and ammoniacal silver carbonate there remains, however, the important difference that the solution of ammoniacal silver carbonate is very heavily buffered, while the other is only weakly buffered.

THE BEHAVIOR OF SILVER SOLUTIONS TOWARD TISSUES AND IN FORMALDEHYDE REDUCTION.

It is readily demonstrated that the three ammoniacal silver solutions show great differences in activity and stability. Of the three, the ammoniacal silver nitrate is the most stable, least sensitive to light, least readily reduced, and least easily combines with tissues. The ammoniacal silver hydroxide solution is at the other extreme in all of these characteristics; the carbonate exhibits properties which lie between these two extremes. These facts are brought out quantitatively in table 6.

The precipitate which forms slowly under the action of formaldehyde on ammoniacal silver nitrate solutions is a slowly spreading cloud of finely divided gray dust which settles gradually and forms on the glass dish a delicate mirror surface. The solution of ammoniacal silver carbonate precipitates more promptly as a darker and heavier cloud and forms more extensive mirror deposits. The solution of ammoniacal silver hydroxide precipitates almost instantaneously as a heavy black cloud and gives inconstant mirror surfaces on the walls of the container.

If these same solutions are heated carefully and slowly to a temperature of 50°C., in the presence of frozen sections of a brain which has been fixed in a solution of formaldehyde-ammonium-bromide (as for the glia stains of Cajal or Del Rio-Hortega), similar differences are seen. The solution of ammoniacal silver nitrate will stain the

sections little or not at all, requiring from thirty-five to forty-five minutes of heating for whatever staining does occur, and in subsequent formaldehyde reduction giving largely a superficial precipitate and little union with the tissue elements. The carbonate begins to stain the sections within five or ten minutes, giving to them a rapidly deepening tobacco tint before the solution itself begins to exhibit any discoloration, and attaining an optimum depth of color in the tissues while the solution still remains pale amber. In subsequent reduction by formaldehyde the tissue elements may show beautiful stains with no diffuse precipitate. The hydroxide is even more rapid in its action. It begins to combine with the sections almost at once, coloring them brown, but almost as quickly the solution darkens and a precipitate starts to form.

THE CORRELATION BETWEEN THE DIFFERENCES IN ACTIVITY AND IN ALKALINITY OF THE SILVER SOLUTIONS.

It is well known that silver nitrate cannot be reduced in an acid solution and is readily reducible in alkali. The theoretical basis for this involves considerations which lie beyond the scope of the present study. But the observations presented here indicate that the same general relationship holds true for the ammoniacal silver solutions; namely, that the more strongly alkaline such a solution is, the more sensitive it becomes to reducing influences. This explains the extreme sensitivity of the solution of ammoniacal silver hydroxide.

The effect of the addition of carbonates to a solution of ammoniacal silver nitrate is explicable on the same basis. Not only are almost all formaldehyde solutions acid,[10] but in the reaction itself in which silver is reduced, formaldehyde is oxidized to formic acid, and nitric acid is produced. The presence of these acids blocks the reduction of the ammoniacal silver nitrate solution. The addition of carbonates to this solution, on the other hand, buffers it against the action of the acids, stabilizes its hydroxyl ion concentration, and thus assists the reduction.

10. Even when made up from so-called "neutral" formaldehyde, absorption of carbon dioxide and spontaneous oxidation to significant amounts of formic acid rapidly produce a pH which ranges from 3 to 5.

TABLE 6.

*Sensitivity to Formaldehyde Reduction and Reactivity with Tissues.**

Solutions tested	Latent Period Before Appearance of Reduced Silver Precipitate in Formaldehyde Solutions of Different Strengths				Latent Period Before Tissue Sections Show Macroscopic Staining
	0.5 Percentage	0.05 Percentage	0.01 Percentage	0.005 Percentage	
		sec.	*sec.*	*min.*	*min.*
$AgNO_3$ plus NH_3 alone	10–15 sec.	90	200+	6+	35–45
				sec.	
$AgNO_3$ plus equivalent NaOH plus NH_3	Instantaneous	2–3	12–15	35–40	4–6
$AgNO_3$ plus equivalent Na_2CO_3 plus NH_3	4 sec.	30–40	110–125	250	15–20
$AgNO_3$ plus equivalent NaOH with washing of the precipitate	Instantaneous	2–3	12–18	38–40	4–6
Idem but without washing precipitate	Instantaneous	2–3	12–15	35–40	4–6
$AgNO_3$ plus equivalent Na_2CO_3 with washing of the precipitate	4 sec.	35–40	115–155	240–260	15–20
Idem but without washing precipitate	4 sec.	30–40	110–125	250	15–20
$AgNO_3$ plus ½ equivalent NaOH	Instantaneous	4.5–5	20–30	45–55	7–9
$AgNO_3$ plus 1 equivalent NaOH	Instantaneous	2–3	12–15	35–40	4–6
$AgNO_3$ plus 2 equivalent NaOH	Instantaneous	1	5–8	12–18	3–5
$AgNO_3$ plus 1 equivalent Na_2CO_3	4 sec.	30–40	110–125	250	15–20
$AgNO_3$ plus 2 equivalent Na_2CO_3	4 sec.	25–35	100–115	.....	15–20
Bielschowsky silver method	Instantaneous	1.5–2.0	6–8	15–25	3–5
Cajal silver method (i.e., Bielschowsky silver method with washing of precipitate)	Instantaneous	2.5–3.0	8–10	25–35	5–6
Hortega silver method	3 sec.	20–30	80–105	200–240	13–19

* The data on formaldehyde reduction were obtained by blowing 0.3 cc. of the silver solution suddenly and vigorously into a small glass dish containing 2 cc. of the formaldehyde solution, and timing with a stop-watch until the moment of appearance of a diffuse cloud. The range of values given for any one reduction expresses both the observational error and the deviations of several observations.

The data on staining of tissues were obtained on the washed sections from a brain which had been fixed in a solution of formaldehyde-ammonium-bromide and cut on the freezing microtome. In each test, several such sections were

The same holds true for spontaneous reduction of the silver solutions, only to a less striking extent. In this case, the only acid involved is the production of nitric acid in the silver solution as the reduction occurs. If this is not absorbed by a buffer, further reduction is lessened. If there is some alkaline buffer salt present, the effect of the nitric acid is blocked.

The nature of the reaction between formaldehyde-fixed brain substance and the silver salts is still so obscure that theoretical treatment is not possible at present. The observations presented in this study show, however, that the reactions of the ammoniacal silver solutions with weak formaldehyde solutions and their reactions with tissues run parallel courses; which suggests that, in some way, the same fundamental laws must apply to both.

THE CHOICE AND PREPARATION OF THE AMMONIACAL SILVER SOLUTIONS.

It has been shown that the three chief methods of preparing ammoniacal silver salts that have been recommended by histologists yield staining solutions which in their fundamental physicochemical properties are alike, but which differ in sensitivity, rate, intensity and stability. These differences have an important bearing on the utility of the various solutions.

For practical purposes, the ammoniacal silver nitrate solution is only rarely valuable. It can be used occasionally instead of silver nitrate as the preliminary bath for a Bielschowsky or Cajal stain for neurofibrils. The sections may then be passed *without washing* into a solution of ammoniacal silver carbonate or ammoniacal silver hydroxide, because the ammoniacal silver nitrate will not give a

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placed in a covered glass dish containing 7 cc. of the silver solution to be tested, and with continual stirring were warmed gently over a pilot flame from a bunsen burner. By avoiding draughts and keeping the height of the pilot flame constant, a quite slow and unvarying rate of warming was maintained; when a temperature of about 50°C. was reached, the temperature was held at that point until the sections were stained to match a light tobacco-brown color standard. (Histologic studies of much of this material have also been made. The later steps of washing and reduction, however, introduce new questions, so that an analysis of the full staining process lies beyond the scope of the present paper and must be reserved for a later report.)

precipitate in these solutions as does silver nitrate. This eliminates the washing between the two silver solutions which is necessary in the usual procedure, and which results, at times, in incomplete impregnations. If ammoniacal silver nitrate is used, the initial bath must be much shorter than the usual bath in silver nitrate.

The ammoniacal silver hydroxide and the ammoniacal silver carbonate can be used interchangeably if the differences in the sensitivity of the two solutions are compensated for by varying their concentrations, the duration of the bath, the heat used, the concentration of the reducing agent, etc. As already indicated, however, the carbonate solution has the double advantage that its hydroxyl ion concentration is not high enough to render it as unstable and as oversensitive as the ammoniacal silver hydroxide, while the presence of the buffer salts makes it possible for the reduction of the carbonate solution to go forward steadily despite the accumulation of acids in the reaction. For this reason, it is easier with the carbonate solution to avoid overstaining and the formation of superficial precipitates.

Preparation of these solutions with reasonable accuracy is so easily achieved that it seems unnecessary to use any longer the traditional inaccurate methods of histologists. The use of more exact and equally simple procedures has the great advantage that it makes it possible for the histologist to turn to the chemist for assistance without having to retranslate all of his roughly prepared solutions into terms with which the chemist can work. For this reason, simple methods of exact preparation of these solutions are outlined:

Silver Nitrate.—As already explained, because it is approximately of the same strength as that customarily used, a 0.6 molar solution of silver nitrate is recommended. ($AgNO_3$, molecular weight $170 \times 0.6 = 102$ Gm. per liter, or 10.2 Gm. per hundred cubic centimeters.) This can be weighed out with an accuracy of 0.1 Gm. on an ordinary chemical balance on filter paper, and does not require a glass-inclosed quantitative balance. It should be transferred to a glass funnel in the mouth of a hundred cubic centimeter volumetric flask. (A graduated cylinder is not sufficiently accurate for this.) By slowly pouring distilled water through the silver nitrate, it can be washed into the flask; after solution is complete, the flask can be filled to the one hundred cubic centimeter mark, thoroughly shaken, and the contents can be transferred to a dark bottle.

Ammonia.—The formula of the ion, $Ag(NH_3)_2^+$ makes it desirable to use a solution of ammonia of twice the molar concentration of the silver nitrate, i.e., 1.2 molar.

(*a*) The molecular weight of ammonia is 17.

1.2 molar = 17 × 1.2 = 20.4 Gm. per liter, or 2.04 Gm. per hundred cubic centimeters.

(*b*) A usual strength of the commercial stock solutions is 28 per cent at a specific gravity of 0.90. (The data are usually on the bottle or can be secured from the manufacturer.) The stock solution used in the present investigation contained, therefore, 0.28 × 0.90 = 0.252 Gm. ammonia per cubic centimeter.

(*c*) $\frac{2.04}{0.252}$ = 8.09 cc. of the concentrated stock solution; diluted to a volume of one hundred cubic centimeters with distilled water, gives a final solution of 1.2 molar.

The concentrated stock solution should be measured in an accurate ten cubic centimeter pipet, or in a microburet, and after transferring to a one hundred cubic centimeter volumetric flask should be diluted to the one hundred cubic centimeter mark.

Sodium Hydroxide.—The equation, $AgNO_3 + NaOH = AgOH + NaNO_3$, makes it evident that a 0.6 molar solution of sodium hydroxide will be chemically equivalent to 0.6 molar silver nitrate. This can best be prepared in the following way:

A strong stock solution of sodium hydroxide is prepared containing 50 Gm. of sodium hydroxide dissolved in one hundred cubic centimeters of water. Actually, however, even dry sticks of chemically pure sodium hydroxide contain significant amounts of carbonate. The amount of this contamination is indicated on the label of the bottle, and a corresponding excess over 50 Gm. of the commercial sodium hydroxide must be weighed out to allow for this. After the solution has cooled, it should be filtered through glass wool or centrifugalized, in order to rid it of the carbonates which are insoluble in alkali of this concentration. The solution should then be stored in a paraffined flask and well stoppered. It will then be stable and can be used repeatedly.

Such a solution of sodium hydroxide, 50 per cent by weight, has a density of 1.53. Each cubic centimeter contains, therefore, 1.53 × 0.50 = 0.77 Gm. of sodium hydroxide.

The molecular weight of sodium hydroxide is 40. A 0.6 molar solution will contain, therefore, 0.6 × 40 = 24.0 Gm. per liter, or 2.4 Gm. per hundred cubic centimeters. Hence, to prepare from the concentrated stock solution, a solution of 0.6 molar sodium hydroxide (which contains 0.024 Gm. per cubic centimeter), it is necessary to take of the stock solution $\frac{0.024}{0.77}$ = 0.0312, or 3.1 cc., and dilute to a total volume of one hundred cubic centimeters.

Once the concentrated stock solution has been prepared, this is the easiest and most accurate method of preparing the sodium hydroxide solution.

Sodium Carbonate.—$2AgNO_3 + Na_2CO_3 = Ag_2CO_3 + 2NaNO_3$. Therefore,

a 0.3 molar solution of sodium carbonate will be equivalent to a 0.6 molar silver nitrate.

Sodium carbonate is obtained in three forms: as the dry compound, the molecular weight of which is 106; as sodium carbonate monohydrate ($Na_2CO_3 \cdot H_2O$), with a molecular weight of 124, as sodium carbonate decahydrate ($Na_2CO_3 \cdot 10OH_2O$), with a molecular weight of 286. Either the dry compound or the decahydrate may be used, as they are relatively stable. If the dry compound is used, it should be purchased in small bottles and should be kept tightly stoppered. The top layer of the salt should always be scraped off and discarded before weighing out a sample, as it will absorb moisture from the atmosphere and the hydrated compound will contain less actual salt in the amount weighed than has been calculated.

For the dry salt, one should use: $0.3 \times 106 = 31.8$ Gm. per liter, or 3.2 Gm. per hundred cubic centimeters.

For the decahydrate: $0.3 \times 286 = 85.8$ Gm. per liter, or 8.6 Gm. per hundred cubic centimeters.

The presence of a certain amount of bicarbonate in the sample of carbonate will not introduce an inaccuracy of sufficient magnitude to require any corrections. These compounds should be dissolved gradually, allowed to cool, made up to final volume in a one hundred cubic centimeter volumetric flask, and transferred to a paraffined flask.

In using the solutions which are prepared in the manner described, it is possible to secure exact equivalents merely by taking equal volumes. If a ten cubic centimeter pipet, graduated to 0.1 cc. is used for the silver nitrate, the sodium hydroxide and the carbonate, and if either a similar pipet or else a fifty cubic centimeter buret similarly calibrated is used for the ammonia, it is possible to keep exact records of the composition of the solutions. The ionic composition of such solutions can then be calculated or determined, so that accurate information will become available for the standardization of these methods.

The most desirable method of preparing the staining solutions is as follows:

1. Use a fifty cubic centimeter or one hundred cubic centimeter glass-stoppered volumetric flask, depending on the concentration of the solution which is finally desired.
2. Into this flask introduce 10 cc. of the 0.6 molar silver nitrate with a ten cubic centimeter pipet.
3. Add ammonia from the buret until complete solution of the silver has occurred. Add ammonia rapidly at first, but shake vigorously between additions toward the end point.
4. Add 10 cc. of the 0.6 molar sodium hydroxide or else 10 cc. of the 0.3 molar sodium carbonate. If the carbonate is used, no more ammonia will be needed.

If the sodium hydroxide is used, more ammonia will be necessary. After this procedure has been carried out once or twice, however, it will be possible to add enough ammonia in the first place to prevent the formation of any precipitate with the sodium hydroxide.

There is a definite advantage in adding the ammonia before the sodium hydroxide in sufficient quantity precisely to prevent the formation of any precipitate by the strong alkali. The precipitated silver oxide, in the presence of a strong base, tends to reduce with the deposition of metallic silver. This metallic silver cannot be returned to solution by the addition of ammonia, and the presence of these granules mixed with the precipitate of silver oxide obscures the end point and leads to the use of a greater excess of ammonia than is necessary. If enough ammonia is used to begin with, there need never be any precipitate formed in the presence of the strong alkali.

Finally, the solution is diluted by the addition of distilled water to the volume desired.

SUMMARY.

1. The ammoniacal silver solution which is prepared from silver nitrate and ammonia alone is spoken of as the solution of "ammoniacal silver nitrate," with sodium carbonate and ammonia as "ammoniacal silver carbonate," and with sodium hydroxide and ammonia as "ammoniacal silver hydroxide."

2. The ammoniacal silver ion which is formed in the three methods used for preparing silver staining solutions is constant in formula, $Ag(NH_3)_2^+$.

3. The activity of this ion is, however, variable, depending on the ions with which it is associated in solution. The variation of activity is not qualitative, but solely one of sensitivity, stability, rate and intensity.

4. It was found that the amount of ammonia required to cause complete solution of the silver compound varied with the hydroxyl ion concentration of the solution, and that the ammoniacal silver nitrate and the ammoniacal silver carbonate required practically the same amount of ammonia, while the ammoniacal silver hydroxide required much more. The ammoniacal silver nitrate and carbonate solutions have, therefore, practically identical hydroxyl ion concentrations, while that of the ammoniacal silver hydroxide solution is much higher.

5. The ammoniacal silver carbonate solution differs from the ammoniacal silver nitrate, however, in having a much higher titratable or reserve alkalinity as shown by titration with acid. This is due to the buffering action of the carbonate salts.

6. The differences in activity of the ammoniacal silver solutions are shown to correlate with these differences in hydroxyl ion concentration and in titratable alkalinity.

7. Consequently, of the three solutions the ammoniacal silver hydroxide, because of its extremely high hydroxyl ion concentration, is the most unstable—that is, it reduces most readily under the action of light or of formaldehyde, and combines most rapidly with tissue elements. The ammoniacal silver nitrate solution is, on the contrary, most "stable" in all of these respects, because its hydroxyl ion concentration is lower, and because the presence of acid in the formaldehyde and the production of acid in the process of reduction arrests the reduction itself. The ammoniacal silver carbonate is also less unstable than the hydroxide because, like the ammoniacal silver nitrate, its hydroxyl ion concentration is lower; but, unlike the nitrate solution, it is readily reduced because the carbonate salts buffer it effectively against the inhibiting action of the acids.

The ammoniacal silver carbonate solution is thus seen to occupy an optimal position, intermediate between the other two.

8. The ammoniacal silver nitrate solution is only rarely useful. Both the hydroxide and the carbonate solutions, on the other hand, have many uses. They are not interchangeable, however, without taking into account the differences in speed and sensitivity of their action. If these differences are compensated for by appropriate adjustment of other steps (such as the concentration, duration, and temperature of the silver baths, and the strength of the reducing agent), the hydroxide and the carbonate solutions can be made to achieve approximately the same results.

9. For most purposes, the ammoniacal silver carbonate solution, because of its greater stability and its somewhat slower action, is more easily handled; certainly, for those who are relatively inexperienced with these methods, it will yield satisfactory stains more readily than the ammoniacal silver hydroxide.

10. Simple and yet accurate methods for preparing these solutions are described, to take the place of the unstandardized methods in customary use.

11. It has been shown that washing the precipitate, after using an excess of sodium hydroxide, only partially removes the excess base, making it preferable to use only equivalent amounts of reagents in the first place.

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THE COLORIMETRIC DETERMINATION OF LIPOID PHOSPHORUS IN BLOOD.

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(Received for publication, February 28, 1928.)

The colorimetric determination of phosphorus published by Briggs[1] lends itself to the determination of lipoid phosphorus without the oxidation of the organic substances present. In the following procedure the lipoid phosphorus is converted by hydrolysis with the H_2SO_4 present in Briggs' molybdate solution into the inorganic form of H_3PO_4. At the same time, the blue color of reduced molybdate is developed. The small amount of lipoid material remaining does not interfere with the reading of the color developed against the standard.

Procedure.—1 cc. of oxalated blood, serum, or plasma is spread on fat-free filter paper. The filter paper is cut in strips of $1\frac{1}{4} \times 7$ inches. The blood is dried in an electric oven at 50°. The strip containing the dried blood is then placed in a Folin sugar tube into which have been introduced about 4 cc. of chloroform. A test-tube, through which water is allowed to syphon, serves as a condenser. As many units as necessary may be connected (Fig. 1). The chloroform is then refluxed on the water bath at 75° for 3 hours. After the extraction is complete, the chloroform is transferred to a 50 cc. volumetric flask, diluted with 20 cc. of distilled water, and 3 cc. each of molybdate and hydroquinone solutions added.[2] The volumetric flask is placed in the water bath at 100° for 30 minutes, at which time the color is fully developed, and after cooling is read against the standard. This

[1] Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

[2] *Molybdate Solution.*—5 per cent ammonium molybdate in 5 N H_2SO_4. Dissolve 25 gm. of ammonium molybdate in 300 cc. of H_2O, add 75 cc. of concentrated H_2SO_4, and dilute with 125 cc. of H_2O. *Hydroquinone Solution.*—Dissolve 5 gm. of hydroquinone and 25 gm. of $KHSO_3$ in 500 cc. of H_2O. *Standard Phosphorus Solution.*—Dissolve 219.3 mg. of KH_2PO_4 in 200 cc. of water and make up to 1 liter. 1 cc. of standard solution is equivalent to 0.05 mg. of phosphorus.

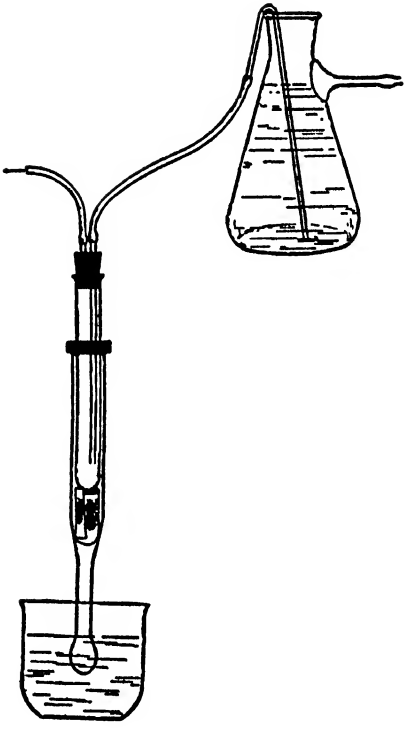

FIG. 1.

TABLE I.

Results are expressed in mg. of phosphorus per 100 cc. of blood.

Blood sample No.	Evaporation of $CHCl_3$ and oxidation with H_2SO_4 and HNO_3	Author's modification.
1	4.63	4.68
2	4.01	4.05
3	4.01	4.16
4	4.17	4.21
5	3.98	4.23
6	4.14	4.21
7	5.03	5.13
8	4.31	4.41
9	4.26	4.32
10	3.94	4.04

procedure eliminates the evaporation of the chloroform extract to dryness.

By making up the chloroform extract to 5 cc. and taking an aliquot part for the determination of phosphorus, cholesterol may be determined on the remaining portion.

Table I gives a comparison of results obtained from ten rabbit bloods.

The results thus far obtained indicate that when applied to the determination of lipoid phosphorus on small amounts of blood, the method described gives results that are as accurate as those obtained by other methods now in use. In addition, there is a great saving of time and material.

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THE CRYSTAL STRUCTURE OF POTASSIUM HYDROXYSTANNATE, $K_2Sn(OH)_6$.

BY RALPH W. G. WYCKOFF.

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INTRODUCTION.

The crystal structures of K_2PtCl_6 and several other cubic crystals isomorphous with it have been known for some time. Another series of compounds of the same chemical type possesses rhombohedral symmetry. Their rhombohedral angles frequently do not depart far from 90° and in habit of growth they closely resemble their cubic analogues. Though most of them do not form large single crystals, potassium hydroxystannate is one of the few that crystallize well.

The $K_2Sn(OH)_6$ needed for the following study was made[1] by fusing 16 grams of KOH in a silver crucible and slowly adding four grams of SnO_2 to the melt. After heating to the decomposition point of KOH the molten mass was cooled and extracted with water. The filtered caustic solution was evaporated to such a concentration that a few crystals formed upon cooling. This solution when allowed slowly to cool from reheating or to desiccate for several days in the open air deposited large clear crystals of $K_2Sn(OH)_6$. These octahedral crystals superficially resemble the cubic chlorostannates and chloroplantinates. They are optically uniaxial, however, and usually the large face upon which they rest is not the base.

Goniometric measurements[2] have shown these crystals to be rhombohedral. The customarily chosen axial angle is $\alpha = 70° 1'$. The observed face development is that of 3Di, the holohedry of the rhombohedral division of the hexagonal system. Density determination[3] has given $\rho = 3.197$.

[1] Marignac, Ann. Min. [5], **15**, 278, 1859.
[2] Zambonini, Zeit. f. Krist. **41**, 53, 1906.
[3] Ordway, this Journal **40**, 173, 1865.

THE CRYSTAL STRUCTURE OF $K_2Sn(OH)_6$.

Two series of Laue photographs have been made, one with the X-ray beam more or less parallel to the trigonal axis, the other with it nearly perpendicular to a rhombohedral face (100). Spectra have been obtained from these same faces. An accurate determination of the size

TABLE I.

The Measured Spacings of the (111) Reflections Observed on a Comparison Photograph of $K_2Sn(OH)_6$.

Line	Order	Spacing	d_{111}/n
β	1n	4.238Å	4.238Å
α_1	1n	4.259	4.259
β	3n	1.416	4.248
α_1	3n	1.418	4.254
α_2	3n	1.416	4.248
α_1	4n	1.063	4.252
α_2	4n	1.063	4.252
Average d_{111}/n.....			4.250Å

TABLE II.

The Average Values of d_{111}/n for $K_2Sn(OH)_6$ as Found from Several Comparison Photographs.

Photograph No.	d_{111}/n
1	4.241Å
2	4.250
3	4.249
4	4.245
	4.24_6Å

of the unit cell is provided by comparison spectra between the cleavage face of calcite and the (111) face of $K_2Sn(OH)_6$. Spacing data from a single photograph are recorded in Table I. The average results from several such spectra are to be found in Table II.

Crystals isomorphous with the point group 3Di can be built upon either a rhombohedral or an hexagonal lattice. The application of

the usual criterion[4] for distinguishing between these two lattices to the numerous data upon the Laue photographs points unmistakably to the correctness of Γ_{rh} for $K_2Sn(OH)_6$.

The number of molecules, m, associated with a rhombohedron of angle α is connected with the spacing d_{111} of the plane (111) and the absolute mass, M, of the molecule by the expression

$$m = \frac{\rho d^3_{111} \times 3\sqrt{3}}{M} \cdot \frac{1 - \cos\alpha}{1 + 2\cos\alpha}$$

TABLE III.

Typical Laue Data from a (111) Photograph of $K_2Sn(OH)_6$.

Indices	Intensity	Spacing	nλ
$1\bar{2}2$	s	1.534Å	0.475Å
$\bar{3}23$	f	.986	.440
$50\bar{1}$	m	.955	.439
$15\bar{2}$	m^+	.881	.387
$\bar{2}50$	f	.873	.367
$0\bar{2}5$	s	.873	.480
$4\bar{3}2$	f	.873	.353
$25\bar{2}$	m	.863	.483
$\bar{1}25$	ff	.842	.345
$43\bar{3}$	f	.821	.287
$2\bar{3}5$	m^+	.773	.448
$4\bar{3}4$	ff	.759	.478
$\bar{2}61$	ff	.759	.471
$63\bar{2}$	s	.730	.475
$\bar{1}\bar{2}6$	f	.728	.357
$\bar{4}34$	ff	.727	.338
$1\bar{3}6$	f	.697	.390
$7\bar{2}2$	m	.665	.448
$0\bar{2}7$	ff	.658	.429

By introducing the known quantities into this equation it is found that $m = 1.008$. Such a cell obviously contains one molecule of $K_2Sn(OH)_6$.

Its correctness is best tested by reference to the Laue data. Since with the voltage used in preparing these Laue photographs the K-absorption edge of tin lies in the region of first order reflection, two

[4] E. Schiebold, Leipziger Abh. 36, 65, 1919; R. W. G. Wyckoff, this Journal 50, 317, 1920.

such tests are possible. First order reflections appear with all sorts of indices and with values of $n\lambda$ down to but not less than the low wave length limit of the X-ray beam. Likewise reflections having $n\lambda$ immediately less than the absorption edge of tin must be very faint. The typical Laue data of Table III and the several hundred first order reflections found upon the analysed photographs all meet these requirements.

Two space groups with the symmetry of 3Di are built upon Γ_{rh}. Only one of them, 3Di-5, has the equivalent positions necessary for placing one $K_2Sn(OH)_6$ within the unit cell. These are:[5]

Sn atom: 000.
K atoms: *uuu; ūūū.*
O atoms: (f) *uū0; ū0u; 0uū; ūu0; u0ū; 0ūu.*
(g) *uū½; ū½u; ½uū; ūu½; u½ū; ½ūu.*
(h) *uuv; uvu; vuu; ūūv̄; ūv̄ū; v̄ūū.*

Were the scattering powers of the oxygen atoms great compared with those of the tin and potassium atoms, it would be necessary to conclude that the correct structure was one of the three deducible from the foregoing special positions of 3Di-5. This is not true, however, and inspection shows that in a two-molecule structure arising from 3Di-6 the tin and potassium atoms have the same relative positions as in the arrangements from 3Di-5:

Sn atoms: (a) 000; ¼¼¼ or (b) ¼¼¼; ¾¾¾.
K atoms: (c) *uuu; ūūū;* $\frac{1}{2} - u, \frac{1}{2} - u, \frac{1}{2} - u; u + \frac{1}{2}, u + \frac{1}{2}, u + \frac{1}{2}$.
O atoms: The twelve general positions of 3Di–6.

The same negligible diffracting power of the oxygen atoms which makes it impossible to select between these five different atomic arrangements permits an accurate determination of the positions of the potassium atoms. Thus it is found that all of the important intensity relationships on the Laue photographs can be explained if the hydroxyl groups are entirely neglected. Provided this is done the usual structure factor becomes

$$A = Sn + 2K\cos 2\pi\, nu(h + k + l); B = 0$$

[5] R. W. G. Wyckoff, An Analytical Expression of the Results of the Theory of Space, p. 157 (Washington, 1922).

where h, k and l are the indices of reflections referred to the one-molecule cell.

The observed intensities of the first four orders from the base are (1) strong, (2) very faint, (3) faint, (4) present. The corresponding orders from (100) are (1) medium strong, (2) very faint, (3) medium, (4) medium. The intensities from (100) are to be explained only if u lies between 0.20 and 0.33. Those from (111) are compatible only with a very narrow region about $u = 0.25$.

The very numerous Laue data provide an excellent means of restricting more narrowly the potassium parameter and of checking the assumption that the oxygen atoms may be neglected. In accord with this assumption it is found that, other factors being equal, all planes with the same value of $(h + k + l)$ have the same reflecting powers. A comparison between planes with different values of $(h + k + l) = \Sigma h$ shows that the following relationships are maintained throughout all the Laue data:

$$\begin{array}{ll} \Sigma h = 3 >> \Sigma h = 2, & \Sigma h = 4 >> \Sigma h = 2 \\ \Sigma h = 4 > \Sigma h = 3, & \Sigma h = 3 > \Sigma h = 5 \\ \Sigma h = 4 > \Sigma h = 5, & \Sigma h = 4 > \Sigma h = 6 \\ \Sigma h = 7 >> \Sigma h = 5, & \Sigma h = 3 > \Sigma h = 6 \end{array}$$

$$\Sigma h = 4 \overline{\overline{ca}} \Sigma h = 7 \overline{\overline{ca}} \Sigma h = 8, \Sigma h = 10 \overline{\overline{ca}} \Sigma h = 9.$$

From these relative intensities it is clear that $0.25 < u < 0.27$.

If the correct structure is based upon the uni-molecular cell, the oxygen atoms are probably in arrangement 3Di-5 (h). In this case the hydroxyl groups form a distorted octahedron about central tin atoms and the symmetry[6] of the resultant $Sn(OH)_6$ groups is that of 3Di. If the oxygen atoms were in (f) of 3Di-5, all six would lie in a single plane perpendicular to the threefold axis of the crystal. Though such a structure can scarcely be shown to be impossible, previous observations upon crystals similar to $K_2Sn(OH)_6$ make it improbable.

In the two structures arising from the two-molecule cell the oxygen atoms are in general positions with distorted octahedral arrangements about central tin atoms. If these atoms are at 000 and $\frac{1}{2}\frac{1}{2}\frac{1}{2}$, the

[6] P. Niggli, Geometrische Kristallographie des Diskontinuums s. 406 (Leipzig, 1919).

$Sn(OH)_6$ group has the symmetry of 3Ci; if they lie at $\frac{111}{444}$ and $\frac{333}{444}$, it is 3D. Expressed in terms of this larger cell the potassium parameter is of course one half of the $u = 0.26$ previously determined.

Although no selection can be made between these possible structures for crystals of $K_2Sn(OH)_6$, investigation of other members of this large isomorphous group of crystals may lead to such a choice. Meanwhile it can be concluded that the positions of the potassium atoms and of the tin atoms (as centers of $Sn(OH)_6$ groups) are expressed by the following coördinates:

Length of edge of unit rhombohedron: $a_0 = 5.66_9$Å
Angle between axis of unit rhombohedron: $\alpha = 70° 1'$
Potassium atoms at: uuu; $\bar{u}\bar{u}\bar{u}$ where $0.25 < u < 0.27$.
Tin atom at: 000.

This structure is best pictured in terms of a pseudo-cubic cell of greater volume than either of the two possible units and containing four molecules of $K_2Sn(OH)_6$. The length of the edge of this cell is $a'_0 = 8.62_8$Å, the angle between its axes $\alpha' = 97° 51'$. The relation between these axes and those of the one- and two-molecule units is the same as that between the axes of the cleavage rhombohedron of calcite and its one- and two-molecule cells.[7] Except for the slight displacement of the potassium atoms along trigonal axes, these and the tin atoms have the same positions in the distorted cube of angle $97° 51'$ as do the calcium and fluorine atoms in the perfect cube of CaF_2. It is thus apparent that the $K_2Sn(OH)_6$ structure may be considered as a K_2PtCl_6-like arrangement which has been distorted by compression along a threefold axis.

SUMMARY.

The atomic arrangement in the rhombohedral crystals of $K_2Sn(OH)_6$ is a distortion of the K_2PtCl_6 structure. The positions of the potassium and tin atoms can be expressed in terms of the following one-molecule unit:

$$a_0 = 5.66_9\text{Å}; \alpha = 70° 1'.$$

[7] R. W. G. Wyckoff, this Journal 50, 317, 1920; cf. Fig. 1.

Sn atom at 000; K atoms at *uuu*; $\bar{u}\bar{u}\bar{u}$ where $0.25 < u < 0.27$. This cell is developed from the space group 3Di-5. It cannot be determined whether the oxygen atoms are in positions (h) of this group or whether the true cell is a two-molecule unit with the hydroxyl groups in the general positions of 3Di-6.

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THE MOLECULAR SYMMETRY OF ACETONYL PYRROLE.

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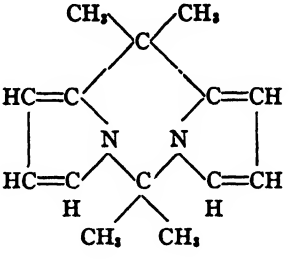
(From The Rockefeller Institute for Medical Research.)

(Received for publication, December 27, 1927.)

The x-ray diffraction patterns obtained from complicated organic compounds have been interpreted in several different ways. The determination of the smallest unit of structure by the use of Laue and spectrum photographs is the only one of these methods not involving questionable assumptions. As a rule a complete structure determination cannot be made but, in most cases, the minimum symmetry of the molecule can be determined. The fixing of this minimum symmetry is of great value in the study of the geometrical configurations of organic compounds.

Acetonyl pyrrole is a condensation product of acetone and pyrrole.[1] C. Liebermann and R. Kraus assigned it the following formula



Condensation supposedly takes place between two moles of acetone and two of pyrrole with the elimination of water, the hydrogen atoms being removed from the N and the α-C atoms. It has been described[2] as crystallizing in the tetragonal system with $a:c = 1:0.8343$.

The material used in this investigation was prepared by heating 10 g.

[1] (a) Baeyer, *Ber.*, **19**, 2184 (1886); (b) Dennstedt and Zimmerman, *ibid.*, **20**, 2450 (1887); (c) C. Liebermann and R. Kraus, *ibid.*, **40**, 2504 (1907).

[2] Fock, *Z. Krist.*, **14**, 541 (1888).

of pyrrole (Eastman) with 15 g. of acetone in 300 cc. of 90% ethyl alcohol containing 4 cc. of concentrated hydrochloric acid.[1] The crystalline material was separated and recrystallized several times from acetone. Crystals several millimeters in length were obtained by

TABLE I.

Typical Laue Photographic Data from Acetonyl Pyrrole.

The incident x-ray beam was approximately normal to (100).

Plane	d_{hkl}, Å	$n\lambda$	Intensity	Plane	d_{hkl}, Å	$n\lambda$	Intensity
$1\bar{6}0$[a]	1.659	0.48	v.w.	1.4.13	1.465	0.45	v.w.
$1\bar{6}2$	1.643	.46	v.w.	$2\bar{9}0$	1.094	.42	v.w.
162	1.643	.38	w.	$2\bar{9}4$	1.076	.41	v.w.
1.I.15	1.548	.46	v.w.	1.3.15[b]	1.431	.43	v.w.
166	1.530	.35	w.	$1.3.\overline{14}$	1.499	.47	v.w.
1.3.14	1.499	.45	v.w.	1.4.14	1.395	.45	v.w.

[a] The indices used in this publication are referred to the space group axes.
[b] Data from a second photograph.

TABLE II.

Typical Spectrum Photographic Data from Acetonyl Pyrrole, Mo K Radiation.

Plane	Line	Order	d_{hkl}, Å	Intensity[a]
001	MoKβ	4	...	s.
	α_1	4	24.30	v.s.
	α_1	8	...	abs.
	α_1	12	23.72	m.
	α_1	16	...	v.w.
100	α_1	2	10.48	
	α_1	3	10.10	
	α_1	4	10.12	
	α_1	5	10.19	

[a] The following abbreviations are used throughout this paper: v.s., very strong; s., strong; m.s., medium strong; m., medium; m.w., medium weak; w., weak; v.w., very weak.

crystallization from acetone solutions containing small amounts of benzene. The developed crystals were elongated octahedra showing evident pyramidal character.

Laue photographs were made with the incident x-ray beam normal to (100) and making small angles with this normal. Data obtained

from one of these photographs are listed in Table I. Spectrum photographs were made on which (100) and (001) reflected as the principal spectrum. These faces were initially parallel to the x-ray beam and were oscillated through an angle of 20° with first the *c*- and then the *b*-axis in the axis of rotation in the former case and the *b*-axis in the latter case. It was very difficult to obtain accurate spacing measurements for d_{100} since it was impossible to determine whether or not reflections from (100) were present. The data listed for (100) in Table II were obtained by measurements to the mid-point of the zone for which h is a constant. In these photographs (100) reflected as the principal spectrum and the crystal, with its *b*-axis in the axis of rotation, was oscillated through an angle of 20°. Spacing measurements were made on reflections from (001), sodium chloride being used as a reference substance (transmission). The spacing d_{001}/n obtained from these measurements is 5.975 Å. in agreement with the value 5.950 Å. calculated from d_{100}/n = 10.09 Å. and the observed axial ratio. The smallest unit of structure compatible with the Laue and spectrum data has $a_0 = b_0 = 10.09$ Å., $c_0 = 23.85$ Å. The density calculated on the basis of this unit containing 8 $C_{14}H_{18}N_2$ is 1.132 in agreement with the density 1.162 determined by the Retgers suspension method.

The observation that a Laue photograph made with the incident x-ray beam normal to (1$\bar{0}$0) shows only a horizontal plane of symmetry requires the structure to be isomorphous with one of the point groups 4*c*, 4*C* or 4*Ci*. Reflections were obtained in the first order from planes having $(h + k + l)$ both odd and even. The underlying lattice is therefore the simple tetragonal one.

The most characteristic feature of the spectrum photographs was the presence of reflections from (001) in only the 4th, 8th, etc., orders (Fig. 1). The only space groups based upon a simple tetragonal lattice and having the symmetry of one of the point groups 4*c*, 4*C* or 4*Ci* explaining this observation are 4*C*-2 and 4*C*-4. The possible molecular centers in these two cases[3] are

4*C*-2 (a) xyz; $\bar{y}$,x,z+½; $\bar{x}$,$\bar{y}$,z+½; y,$\bar{x}$,z+½
4*C*-4 (a) xyz; $\bar{y}$,x,z+½; $\bar{x}$,$\bar{y}$,z+½; y,$\bar{x}$,z+½

[3] R. W. G. Wyckoff, "An Analytical Expression of the Results of the Theory of Space Groups," *Carnegie Inst. Pub.*, No. 318, Washington, 1922, p. 80.

In either case there can be only four equivalent molecules in the unit of structure. It is thus possible that if $C_{14}H_{18}N_2$ is the structural molecule there are two sets of such molecules. It is more probable, however, that the real structural molecule is $C_{28}H_{36}N_4$ corresponding to a condensation product of four moles of pyrrole with four of acetone.

An investigation of the literature showed that the original formula had been assigned without a molecular weight determination being made. For this reason it was necessary to make such a determination.

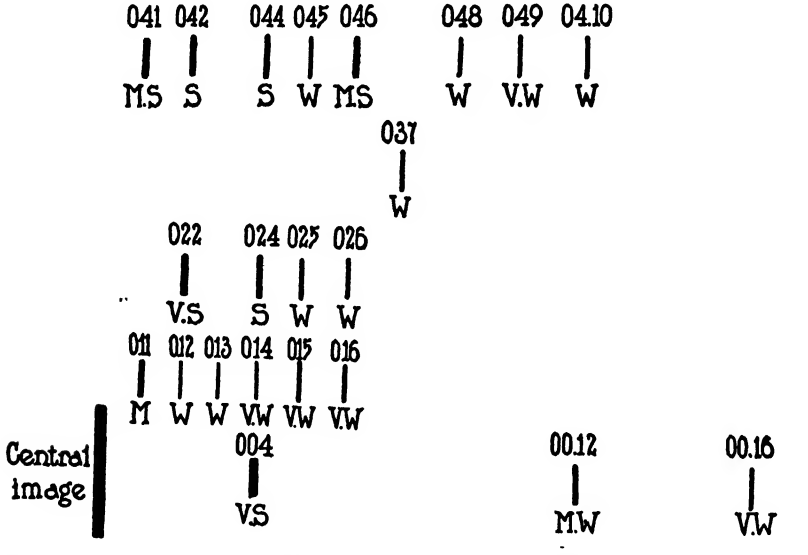


Fig. 1.—A reproduction of a spectrum photograph showing reflection from (001) in the equatorial zone. The *b*-axis of the crystal was vertical. Only the Mo Kα lines are shown; 20° oscillation.

The previously described differential vapor pressure method[4] was used, benzene being used as the solvent; 0.656 g. of acetonyl pyrrole in 100 cc. of the solvent at the boiling point gave an equivalent boiling point elevation of 0.052°, corresponding to a molecular weight of 403. The molecular weight calculated for $C_{28}H_{36}N_4$ is 428. It thus seems very probable that $C_{28}H_{36}N_4$ is the structural molecule. These molecules, since their centers are in the general positions, do not necessarily have an element of symmetry.

[4] Menzies, THIS JOURNAL, 43, 2309–2314 (1921); Menzies and Wright, *ibid.*, 43, 2314–2323 (1921).

The formula $C_{28}H_{36}N_4$ depends upon the analysis of Dennstedt and Zimmerman.[1] It is possible that the hydrogen content of the molecule is not correctly given. Such a possibility does not influence the correctness of the above conclusions concerning the molecular symmetry and the number of molecules associated with the unit of structure.

SUMMARY.

Laue and spectrum photographs have been obtained and analyzed from crystals of acetonyl pyrrole. The unit of structure containing four $C_{28}H_{36}N_4$ molecules has the dimensions $a_0 = b_0 = 10.09$ Å., $c_0 = 23.85$ Å. The space group is $4C$-2 or $4C$-4, the molecules being in the general positions and thus not necessarily having an element of symmetry. The molecular weight is twice that corresponding to the previously assigned formula. A determination of the molecular weight by a differential vapor pressure method gave a value in agreement with the formula $C_{28}H_{36}N_4$.

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THE CRYSTAL STRUCTURE OF LITHIUM IODIDE TRIHYDRATE.

BY STERLING B. HENDRICKS.

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The halides of lithium form hydrates, ammoniates and alkyl ammoniates the regions of stability of which are partially known. The equilibrium diagrams for the systems LiCl-H_2O, LiBr-H_2O and Li-H_2O as determined by G. F. Hüttig and W. Steudemann[1] are shown in Fig. 1. From this figure it can be seen that LiCl.H_2O, LiBr.$2H_2O$ and LiI.$3H_2O$ are the stable phases at room temperatures. The crystal structure of LiCl.H_2O has been determined.[2] The purpose of the present investigation was to determine the crystal structure of LiI.$3H_2O$ or of LiBr.$2H_2O$.

The lithium halide was prepared by the action of the halogen acid upon lithium carbonate. Crystals were grown from the aqueous solutions by desiccation over sulfuric acid. Optical examination of the crystals of LiBr.$2H_2O$ showed them to be biaxial. This compound was not examined further. Crystals of LiI.$3H_2O$ proved to be extremely plastic and deliquescent. It was possible to bend a single crystal through an angle of 270° without producing a visible fracture. Undeformed crystals were uniaxial in character while the deformed ones gave distorted uniaxial figures which could have been confused with biaxial figures. The previous description of this compound as forming optically biaxial crystals[3] might have resulted from an examination of deformed crystals.

The developed crystals were apparently hexagonal prisms elongated in the c direction. A Laue photograph made with the incident X-ray beam normal to one of the prism faces shows a two-fold axis and two planes of symmetry. This observation requires the crystals to be hexagonal rather than tetragonal with an atomic arrangement isomorphous with point group 6d, 6e, 6D or 6Di.

[1] Z. phys. Chem. **126**, 105–117, 1927.

[2] Hendricks, S. B., Zeit. f. Krist. **66**, 297 (1927).

[3] Troost, Ann. Chim. Phys. (3) **51**, 126, 1857.

Laue photographs were made with the incident X-ray beam normal to (010) and making small angles with this normal. Spectrum photographs were made with (010) and (001) as the principal reflecting faces being initially parallel to the X-ray beam and oscillated through an angle of 20° about some crystallographic direction. The smallest

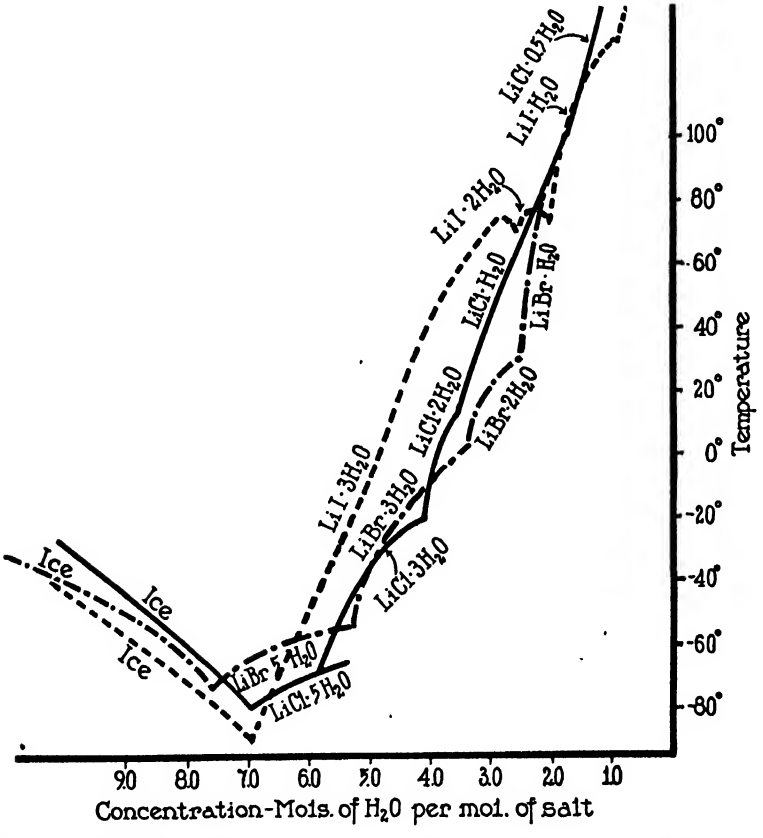

Fig. 1. Equilibrium diagrams for the systems LiX–H_2O (X = Cl, Br, I) after Hüttig and Steudemann.

unit of structure compatible with the Laue data listed in Table II and the spectrum data of Table I and Fig. 2 has d_{100} = 7.45Å and d_{001} = 5.45Å. The density calculated on the basis of such a unit containing two $LiI.3H_2O$ is 2.375. The density experimentally determined by the suspension method of Retgers (bromoform-alcohol mixtures being used) is 2.29. This latter value might be in error due to the difficulty

encountered in drying the crystals and on account of their solubility in the suspending media.

The only space groups isomorphous with point groups 6Di, 6D, 6e or 6d giving possible positions for two LiI.$3H_2O$ in the unit of structure are 6Di-1, 6Di-2, 6Di-3, 6Di-4, 6D-1, 6D-6, 6e-1, 6e-2, 6e-3, 6e-4, 6d-1, 6d-2, 6d-3 or 6d-4. Of the space groups listed above only 6e-4, 6Di-4, and 6D-6 account for the absence of odd order reflections from (001) (Table I) and the observed very low intensity of reflection from (031) and quite strong reflections from (021) and (041) (Fig. 2). The atomic arrangement derivable from 6D-6 with H_2O at (g)[4] would be quite unlikely chemically

(a) $00u$; $0,0,u+\frac{1}{2}$. (b) $\frac{1}{3}\frac{2}{3}u$; $\frac{2}{3},\frac{1}{3},u+\frac{1}{2}$.
(c) $u\bar{u}v$; $2\bar{u},\bar{u},v$; $u,2u,v$;
$\bar{u},u,v+\frac{1}{2}$; $2u,u,v+\frac{1}{2}$; $\bar{u},2\bar{u},v+\frac{1}{2}$.

since it would lead to a $3H_2O$ group not associated with the iodine or lithium atom. The other possible arrangement resulting from 6D-6 or 6Di-4 are special cases of the more general arrangement derivable from 6e-4. The possible arrangements for two LiI.$3H_2O$ in this space group are:

TABLE I.

Typical Spectrum Photographic Data from Lithium Iodide Trihydrate. Mo K Radiation.

Plane	Line	Order	d_{hkl} Å/n	Observed Intensity	$\sqrt{s}$ Calculated for Iodine Atoms
001(1)	mo $K\beta$(2)	2	5.41	m.s.	
	α_1	2	5.45	v.s.	2.00 x I
	β	4	5.41	m.w.	
	α_1	4	5.42	m.s.	2.00
010	β	2	6.48	m.w.	
	α_1	2	6.50	m.s.	1.00
	β	3	6.49	m.	
	α_1	3	6.46	s.	2.00
	α_1	4	6.46	w.	1.00

(1) NaCl was used as a reference substance in the determination of the interplaner spacings for (001).

(2) The following wave lengths were used in the calculations, mo $K\beta$, λ = .6311Å; mo $K\alpha_1$, λ = .7078Å.

[4] Wyckoff, R. W. G., The Analytical Expression of the Theory of Space Groups, p. 166. Washington, 1922.

If two atoms, lithium or iodine, were in positions (a) their maximum distance apart would be 2.72Å, which is improbably small for the iodine atoms. Such an arrangement would amount to an association of ions of like charge in the unit of structure. It is more probable that both the iodine and lithium atoms are at (b) with different values of

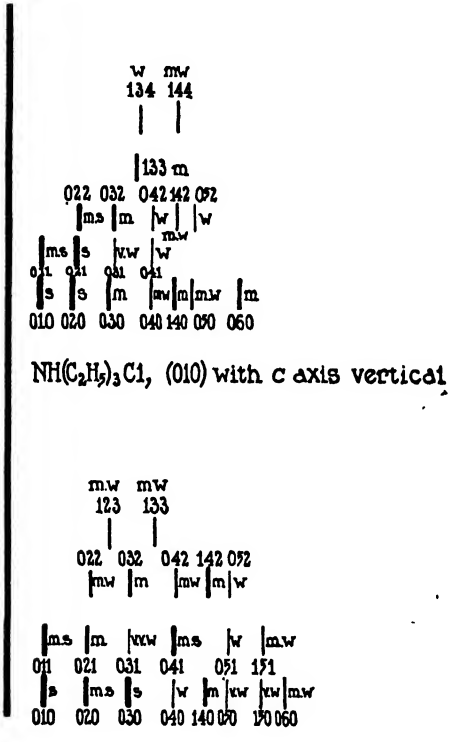

Fig. 2. A partial reproduction of spectrum photographs from similarly oriented crystals of $LiI.3H_2O$ and $NH(C_2H_5)_3Cl$, mo $K\alpha_1$ lines alone being shown.

the parameter. In order to account for the presence of reflection from (031) the oxygen atoms of the H_2O molecules must be at (c).

The extremely low scattering powers of the lithium atoms and oxygen atoms of an H_2O molecule relative to that of the iodine atoms makes an evaluation of the parameters impossible at the present time. The presence of a reflection from (031) of very low intensity (Fig. 2)

indicates that the oxygen atoms do have a slight effect upon the intensity of reflection. In order that the oxygen to oxygen distance should not be less than 1.0Å, u_0 must be between .04–.29. The agreement between the observed intensities of reflection and the calcu-

TABLE II.

Typical Laue Photographic Data from Lithium Iodide Trihydrate. Tungsten General Radiation, 52,000 V Peak.

Plane	d_{hkl} Å	nλ	Observed Intensity	$\sqrt{s}$ Calculated for Iodine Atoms
$\bar{1}13$ (1)	1.742	.47	v.s. (2) (3)	1.73 x I
103	1.742	.47	v.s.	1.73
$\bar{5}30$	1.471	.40	m.	1.00
$3\bar{1}3$	1.451	.32	m.w.—m.	1.73
$\bar{3}23$	1.451	.35	m.w.—m.	1.73
$\bar{5}31$	1.420	.38	m.s.	1.73
$5\bar{2}\bar{1}$	1.420	.29	m.w.—m.	1.73
$4\bar{1}3$	1.269	.47	s.	1.73
$6\bar{2}1$	1.183	.41	m.s.	1.73
$6\bar{2}\bar{1}$	1.183	.42	m.s.	1.73
$\bar{6}41$	1.183	.46	m.s.—s.	1.73
$7\bar{2}0$	1.027	.47	m.s.	2.00
205	1.028	.32	w.	1.73
$\bar{2}25$	1.028	.34	w.	1.73
$\bar{6}43$	1.007	.34	w.	1.73
$\bar{5}44$	.975	.46	m.s.	2.00
$7\bar{2}2$	.961	.41	m.—m.s.	2.00
$\bar{8}51$	.904	.28	v.v.w.	1.73
$6\bar{1}4$	.879	.47	v.w.	1.00
$8\bar{2}1$	.878	.46	w.	1.73

(1) The incident X-ray beam was approximately normal to (010).

(2) The following abbreviations are used throughout this paper; v.s., very strong; s., strong; m.s., medium strong; m., medium; m.w., medium weak; w., weak; v.w., very weak; v.v.w., very, very weak.

(3) It is to be remembered that the K absorption limit of iodine is at λ = .3737Å, for the element.

lated structure factors, iodine atoms alone being considered, is shown in Tables I and II.

It is to be noted in Table II that the reflection from (531) at λ = .38 is of much greater intensity than that from (323) at λ = .35 even

though the planes have approximately the same interplaner distance and the same structure as far as iodine atoms alone are considered. The absorption edge for the element iodine is at $\lambda = .3737$Å. This is further confirmation for the correctness of the determination of the unit of structure.

DISCUSSION.

The unit of structure containing two $LiI.3H_2O$ is shown in Fig. 3. In this figure the H_2O molecules are shown as associated with the lithium atom. It is impossible at the present time to eliminate the structure in which the H_2O molecules would be associated with the iodine atom. In the crystal structure determination of the previously

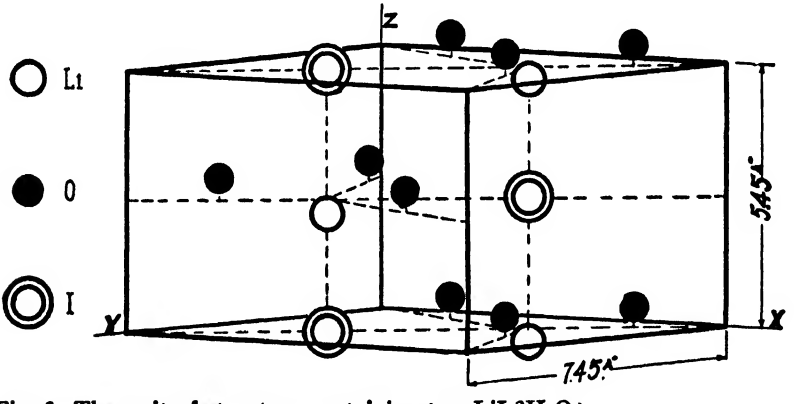

Fig. 3. The unit of structure containing two $LiI.3H_2O$.

studied $LiCl \cdot H_2O$[5] it was definitely shown that the H_2O molecule was not associated with the chlorine atom, although it could not be proven that it was associated with the lithium atom. In the case of $LiI.3H_2O$ it is further possible that v_0 might be about one half of u_{Li} (u_{Li} being taken as zero) which would not require the presence of an $Li.3H_2O$ or an $I.3H_2O$ group in the unit of structure.

The space group and the resulting atomic configuration of lithium iodide trihydrate are the same as that of the previously studied triethyl ammonium halides.[6] This similarity is shown by writing the formula as I (Li $(H_2O)_3$) and I (NH $(C_2H_5)_3$). The lithium atom can

[5] Hendricks, S. B., Zeit. f. Krist. 66, 297 (1927).
[6] Hendricks, S. B., Zeit. f. Krist. (in press).

be considered as equivalent to the (NH) group while each H_2O molecule is equivalent to a (C_2H_5) group. We can thus have an $Li(H_2O)_3$ group which is structurally similar to the $NH(C_2H_5)_3$ group. This marked similarity in structure is indicated by the two spectrum photographs reproduced as Fig. 2.

This similarity between the geometrical configuration of the hydrates of lithium and the alkyl ammonium compounds is further shown in the case of $LiCl.H_2O$[7] and NH_3CH_3Cl,[8] the $(Li.H_2O)$ group being structurally similar to the (NH_3CH_3) group.

The Li-I distance is the only distance that can be considered on the basis of the present data. If the value of u_{Li} is one half, the Li-I distance is ca 2.72Å. In the case of the previously studied anhydrous lithium iodide[9] the Li-I distance is ca 3.00Å if the structure is similar to that of sodium chloride. This distance is in agreement with the value 2.76 recently given by Pauling[10] as the distance to be expected between the centers of spherical Li^+ and I^- ions in contact.

CONCLUSIONS.

The crystal structure of lithium iodide trihydrate has been determined by the use of Laue and spectrum photographs. The hexagonal unit of structure containing two $LiI.3H_2O$ has $d_{100} = 7.45$Å and $d_{001} = 5.45$Å. The space group is 6e-4.

The atomic positions are:

I at (b) $\frac{1}{3}\frac{2}{3}u$; $\frac{2}{3},\frac{1}{3},u+\frac{1}{2}$ (with $u = 0$);
Li at (b) $\frac{1}{3}\frac{2}{3}u$; $\frac{2}{3},\frac{1}{3},u+\frac{1}{2}$; 0 at (c)

$$u\bar{u}v; \quad 2\bar{u},\bar{u},v; \quad u,2u,v;$$
$$\bar{u},u,v+\tfrac{1}{2}; \quad 2u,u,v+\tfrac{1}{2}; \quad \bar{u},2\bar{u},v+\tfrac{1}{2}.$$

It was impossible to determine the values of the parameters.

[7] Hendricks, S. B., Zeit. f. Krist. **66**, 297, 1927.

[8] Hendricks, S. B., Zeit. f. Krist. (in press).

[9] Ott, H., Phys. Zeit. **24**, 209, 1923; Wyckoff, R. W. G., and Posnjak, E. W., J. Wash. Acad. Sci. **13**, 393, 1923.

[10] Pauling, L., J. Am. Chem. Soc. **49**, 765, 1927.

[Reprinted from THE JOURNAL OF IMMUNOLOGY, May, 1928, Vol. XV, No. 3, pp. 283–288.]

THE EFFECT OF CHLOROFORM ON THE IMMUNIZING ACTION OF VACCINE VIRUS.

BY F. DURAN REYNALS.[1]

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 17, 1928.)

In a recently published study from this laboratory (1) it was shown that the infecting power of vaccine virus kept in contact for a short time with chloroform was so reduced that no lesions developed in rabbits inoculated with the usual amounts. If, however, the fluid from "cultures" of embryonic tissue incubated anaerobically in Hartley's broth media was added to the treated virus it gave rise to typical vaccinal lesions and immunity subsequently developed. It was invariably found, whenever it was possible to reactivate the treated virus by the above mentioned fluid, that very large doses of the treated vaccine would alone produce mild but typical lesions. In other words the reactivation of the treated virus was possible only when this material contained a few active elements. Incidental observations were made which seemed to throw some light on the much discussed question as to whether immunity can be induced by killed vaccine virus.

Nakagawa (2) claims that it is possible to produce a local or general immunity by the repeated injection of vaccine virus after it has been heated for forty minutes at 98°C. This author believes that the antigen in this case is in the nature of a water soluble "immunogen" extracted by the treatment from the protoplasm of the phagocytic cells. If this is true the immunity is of a different type from that produced by killed organisms.

In an analysis of the extensive work on this subject, Gordon (3) concludes that "the heating of vaccinia to 70°C. materially reduces, if it does not destroy altogether, the prophylactic value of vaccinia virus." The weight of the published evidence is undoubtedly in favor of the view that when vaccine virus is inactivated by a moderate amount of heat, it still possesses prophylactic properties but the question remains open as to whether all of the virus elements are killed by this treatment.

[1] Fellow of the Spanish Government (Junta para Ampliación de Estudios).

In the course of our study referred to above, as controls, a number of rabbits were inoculated with vaccine virus which had been treated with varying amounts of chloroform. These animals gave us an

TABLE 1.

Rabbit number	Initial treatment			Days between first and second injections	Immunity test		
	Amount of chloroform per 10 cc. vaccine virus suspension	Amount of chloroform vaccine virus injected	Resulting lesion		Amount of normal vaccine virus injected	Resulting lesion	Day of maximum intensity
	cc.	*cc.*			*cc.*		
60	1.0	10.0	0	21	0.4	+++	5
61	0.5	10.0	0	21	0.4	+++	5
57	0.25	8.0	0	23	0.4	+++	4
56	0.2	8.0	0	23	0.4	+++	5
51	0.15	10.0	0	32	0.4	+++	5
54	0.15	10.0	0	30	0.4	++	3
6	0.2	0.2	0	16	0.2	+++	5
7	0.2	0.4	0	16	0.2	+++	5
18	0.15	2.0	+	51	0.2	§	3
30	0.2	6.0	1 small pustule	26	0.2	§	2
28	0.2	2.0	1 small pustule	26	0.2	§	1
31	0.15	6.0	+	26	0.2	+	4
29	0.15	2.0	+	26	0.2	§	3
35	0.15	6.0	++	26	0.2	§	3
36	0.15	7.5	+	26	0.2	§	3
38	0.15	17.0	++	22	0.2	§	3
39	0.15	10.0	+	22	0.2	§	2
40	0.15	12.0	++	21	0.2	§	1
41	0.15	4.0	++	21	0.2	+	1
58	0.15	9.0	Two pustules	20	0.4	−	
59	0.2	8.0	+	20	0.4	−	
16	0.15	10.0	+	31	0.2	§	3
17	0.15	10.0	+	31	0.2	§	3

−, no eruption; §, allergic reaction; +, allergic reaction or abortive eruption; ++, mild positive eruption; +++, strong positive eruption.

opportunity of testing the immunizing power of the virus after such treatment. Some of the rabbits in the series had been inoculated with the virus after treatment with larger amounts of chloroform

when reactivation had proved impossible and when it may be assumed that all of the virus was killed. Other rabbits had been inoculated with attenuated virus known to contain the living agent either from the fact that the addition of "culture" fluid had enabled it to produce lesions in other rabbits, or that the injection of large amounts alone had resulted in slight vaccinal eruptions. In the present experiments the immunity of this series of rabbits was tested by the subsequent injection of untreated vaccine virus.

TABLE 2.

Control for the Immunizing Power of Vaccine Virus.

Rabbit number	Amount of 1 per 10 pure vaccine injected	Resultant lesion	Days between first and second injection	Amount of normal vaccine dilution injected	Resultant lesion	Day of maximum intensity	
	cc.			*cc.*			
42	0.4	++	18	0.2	§	1	First inoculation by injection into the skin
43	1.0	+++	18	0.2	−		First injection into the testicle
37	0.4	+++	22	0.2	−		First injection by scarification of the skin
5	0.4	+++	75	0.2	§	3	First injection by scarification of the skin
33	0.2	+++	5				
55	0.2	+++	5				
62	0.4	++	3				

−, no eruption; §, allergic reaction; +, allergic reaction or abortive eruption; ++, mild positive eruption; +++, strong positive eruption.

Experimental procedure.—The same strain of vaccine virus was used throughout the experiments. It was prepared by removing the testicles of an adult rabbit five days after it had been inoculated with a virulent strain of the virus. This material was ground thoroughly with sand and 25 cc. each of Ringer's solution and glycerine were added. For the experiments this suspension was diluted 1 to 10 with Ringer's solution. The intradermal injection of 0.2 cc. of this dilution gave quite regularly in four or five days a greatly congested eruption 2.5 to 3 cm. in diameter.

Chloroform was added to 10 cc. of the diluted virus, thoroughly mixed, and incubated at 37°C. for seventy-five to ninety minutes. The chloroform was then boiled off in a vacuum at room temperature.

Experiments.—The immunizing power of the vaccine virus after treatment with

varying amounts of chloroform was tested by the intradermal injection of the material in 23 rabbits. The immunity developed as a result was determined by an intradermal injection of the untreated virus, sixteen to fifty-one days later. For controls, the immunizing power of the untreated virus was tested on 4 animals and the virulence of different samples on 3 animals. The results of these tests have been brought together in tables 1 and 2.

It will be seen from table 1 that there was some variation in the effect of the same amount of chloroform on the different samples of the virus. This was to be expected as there was no method of determining the amount of virus present in the different preparations. There is no indication that the virus which had been completely inactivated by the chloroform treatment had any antigenic properties and no immunity developed except in instances in which the first injection induced an eruption, even though in some cases only one pustule resulted. Following the second injection an allergic reaction resulted in several instances and a probably abortive eruption in two of the animals; but it will be noted that of the four control rabbits immunized with the active virus two also showed an allergic type of reaction.

DISCUSSION.

Very feeble skin reactions resulting from small injections of attenuated vaccine virus can not be diagnosed as vaccinal with any degree of certainty by direct examination. As stated in our previous paper (1) the true character of such eruptions can be judged if the virus can be recovered from such a lesion by testicular passage in the rabbit. If the sample of attenuated virus is injected into an area of skin previously irritated, or in much larger amounts into normal skin, typical vaccine eruptions occur. Judging by the results reported above, whenever injections of large amounts of chloroformed virus gave no lesions, immunity failed to develop as shown by a second injection of untreated virus. On the other hand whenever an eruption, even a single pustule, developed after the injection of the treated virus a definite immunity resulted as shown by the fact that the second injection induced either no lesion whatever, a quick allergic reaction or an abortive eruption.

From our experience with reactivated virus, previously reported

(1) it seemed certain that the attenuated virus activated to a point where it produced a lesion, gave rise to immunity; while the same amount, presumably containing as many active units, injected alone and giving rise to no lesion, produced no immunity. This observation strengthens the conclusions to be drawn from the present experiments.

In a recently published paper, Hunt and Falk (4) claim to have obtained immunity against vaccinia by means of repeated injections of virus supposedly killed either by heating at 60°, treatment with weak formaldehyde solution for twelve hours, or by keeping the vaccine in contact with serum of an immunized animal and then heating for one hour at 60°C. The statement that the vaccine virus was rendered inactive by these treatments was based only on the fact that no lesions resulted from the application of the virus to scarified areas on the skin of rabbits. It is well known that this is a much less satisfactory test than either intradermal or testicular inoculations. For this reason we consider that Hunt and Falk, as well as some other investigators dealing with the problem are not justified in considering that they have sufficient proof of the complete destruction of the virus.

There is no evidence to be found in our experiments to support the idea that a soluble product or accessory factor plays a rôle in vaccinal infection or immunity. The results seem to show that both infection and immunity are developed only as result of the action of living virus upon the tissues.

SUMMARY AND CONCLUSIONS.

When vaccine virus was so far inactivated by chloroform that no eruption resulted from the intradermal injection of very large doses, no immunity was induced in the animals.

When the chloroform virus produced any lesion whatever, the rabbits subsequently proved immune when tested by a second injection of untreated virus.

These experiments support the general idea that inactivated vaccine virus is incapable of giving rise to immunity.

REFERENCES.

(1) Duran-Reynals, F.: Jour. Exper. Med., 1928, **47**, 389.
(2) Nakagawa, W.: Ztschr. f. Immunitätsforsch. u. Exp. Therap., Orig., 1924, **39**, 173; ibid., 563; ibid., **42**, 409.
(3) Gordon, M. H.: Studies of Viruses of Vaccinia and Variola. London, 1925 (Med. Research Council).
(4) Hunt, L. W., and Falk, I. S.: Jour. Immunol., 1927, **14**, 347.

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THE METABOLISM OF LIVER TISSUE FROM RATS OF DIFFERENT AGES.

BY JAMES A. HAWKINS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, February 18, 1928.)

Falk, Noyes and Sugiura (1) have studied the lipase or ester-hydrolyzing actions of aqueous extracts of the whole rat at different ages covering the life cycle from 3 days before birth until the age of 3 years, and presented the results in the form of curves. They found that the curves for the embryo and youngest rats approached those given by the Flexner-Jobling carcinoma, changing with the age of the rat to the type characteristic of the adult rat and appearing to revert again to some extent to the embryonic type for the oldest rats. Similar experiments with the protease actions of the extracts of whole rats of different ages on three protein preparations did not give differences similar to those found for the lipase actions.

Warburg (2) and his associates as a result of an extensive study of metabolism of tissues consider that they can detect four distinct types, namely; normal resting tissue with a slight anaerobic glycolysis and a high respiratory rate; embryonic tissue with a high respiratory rate and a high anaerobic but a low aerobic glycolysis; malignant tumor tissue with a low respiration and a high aerobic and anaerobic glycolysis; benign tumor tissue with the same type of metabolism as malignant tissue but with less active glycolytic function. Murphy and Hawkins (3) extended Warburg's experiments and concluded that a classification of tissues on the basis of the type of metabolism does not correspond to the biological groupings but that a classification (4) could be made by measuring the anaerobic glycolytic function of the tissues.

The present experiments were undertaken to determine whether there is any difference in the metabolism of the livers of rats of different ages. The livers of rats in 3 groups were used; Group 1, rats 22

months old; Group 2, adult rats approximately 1 year old; Group 3, rats varying in age from 3 to 21 days old.

The method used was that devised by Warburg, the details of which are described by him in his publications (2) and also by Murphy and Hawkins (3). The results of the experiments are given in Table I.

The results in Table I show that there is no difference in respiration and aerobic glycolysis in the livers of rats of different ages. There is practically no anaerobic glycolytic activity in the livers of the old and normal adult rats, but there is a certain amount in the livers of the

TABLE I.

Average for group	No. of animals	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$
1	15	−11.5	1.0	0.8
2	12	−9.4	0.4	2.0
3	15	−13.2	0.0	5.7

Q_{O_2} = Respiration. C.mm. of oxygen consumed per hour by 1 mg. per dried weight of tissue.

$Q_{CO_2}^{O_2}$ = Aerobic glycolysis. C.mm. of carbon dioxide produced by glycolysis per hour by 1 mg. per dried weight of tissue.

$Q_{CO_2}^{N}$ = Anaerobic glycolysis. C.mm. of carbon dioxide produced per hour by 1 mg. per dried weight of tissue when respiration has been checked by potassium cyanide.

very young rats. This would mean according to Warburg's grouping that the livers of young rats have the same type of metabolism as embryonic tissue. These results would seem to substantiate the conclusions of Hawkins that the glycolytic activity of a tissue is a function of the growth rate, as the growth rate of the livers of the young rats would be greater than that of normal adult or old rats.

Accepting the conclusion that the glycolytic activity of a tissue is a function of its growth rate, these results are in agreement with those of Falk and his associates who found that their curves for embryo and young rats approached those for the Flexner-Jobling tumor.

BIBLIOGRAPHY.

1. Falk, F. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.* 1925–28, viii, 75.
2. Warburg, O., and Minami, S., *Klin. Woch.*, 1923, ii, 776. Warburg, O., Negelein, E., and Posener, K., *Klin. Woch.*, 1924, iii, 1062. Warburg, O., *Biochem. Z.*, 1923, cxlii, 317. Ninami, S., *Biochem. Z.*, 1923, cxlii, 334. Warburg, O., *Biochem. Z.*, 1924, clii, 51. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309. For a resumé see Warburg, O., *J. Cancer Research*, 1925, ix, 148.
3. Murphy, J. B., and Hawkins, J. A., *J. Gen. Physiol.*, 1925–28, viii, 115.
4. Hawkins, J. A., *J. Gen. Physiol.*, 1925–26, ix, 771.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, May, 1928, Vol. lxxvii, No. 2, pp. 555–562.]

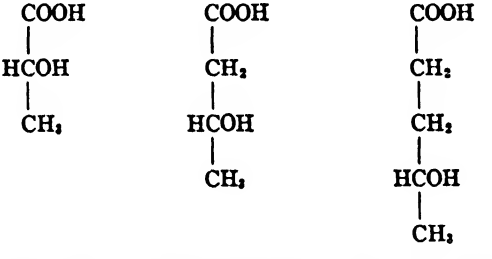
CONFIGURATIONAL RELATIONSHIPS OF 2-HYDROXY-VALERIC AND LACTIC ACIDS.

BY P. A. LEVENE AND H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 10, 1928.)

Until recently the configuration of 2-hydroxypropionic (lactic) acid only had been established by direct chemical methods. The conclusions regarding the configurations of the higher monohydroxy acids were based on indirect methods and such conclusions are never as convincing as those arrived at by direct methods. Our first efforts in this direction resulted in correlating the configurations of 3-hydroxybutyric[1] and 4-hydroxyvaleric acids[2] with that of lactic acid.



Levo-lactic acid.	Levo-3-hydroxybutyric acid.	Levo-4-hydroxyvaleric acid.
I.	II.	III.

This beginning proved very fortunate, for it permitted us to establish the configurations of methylethyl and methylpropyl carbinols.[3] The knowledge of the configurations of these two carbinols made it possible to establish the configurations of 2-hydroxybutyric and 2-hydroxyvaleric acids. The work on the configuration of 2-hydroxybutyric

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxv, 49; 1926, lxvii, 329. Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1926, lxviii, 415.

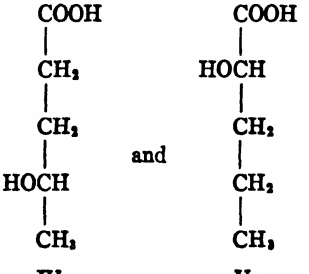
[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165, 569.

[3] Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 591. Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1926–27, lxxi, 465.

acid has been made the topic of a previous communication[4] and the work on the configuration of 2-hydroxyvaleric acid is presented here.

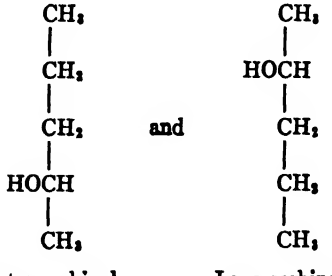
The reasoning which led to the conclusion regarding the configuration of 2-hydroxyvaleric acid is analogous to that which served to elucidate the configuration of 2-hydroxybutyric acid.

Taking two hydroxy acids with the hydroxyls on the same side of the carbon chain, but allocated at different distances from the carboxyl, such as



IV. V.

and in each case reducing the carboxyl group to a methyl group, two carbinols which are enantiomorphous to each other will be obtained:



Dextro-carbinol from (IV). Levo-carbinol from (V).

VI. VII.

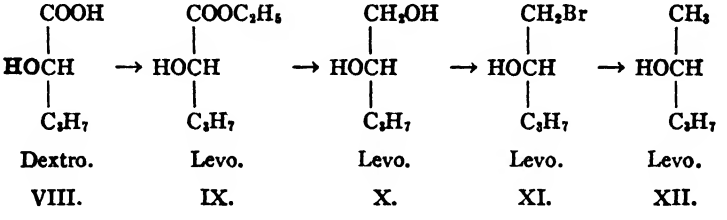
Hence if a given optically active 2-hydroxyvaleric acid is converted into levo-methylpropyl carbinol, this acid has the hydroxyl on the same side of the carbon chain as that of the 4-hydroxyvaleric acid which leads to a dextro-methylpropyl carbinol, namely the dextrorotatory form.

In our case, dextro-2-hydroxyvaleric acid on reduction yielded levo-

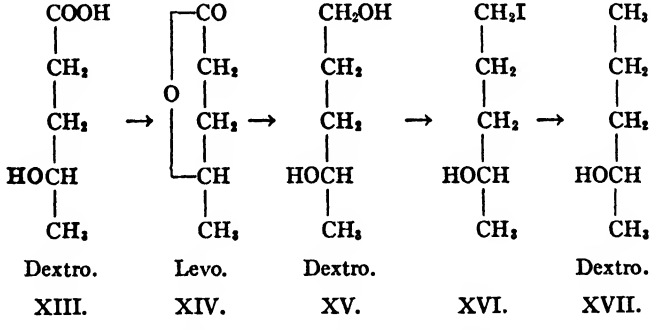
[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxiv, 343.

methylpropyl carbinol; hence in this acid the hydroxyl is allocated on the same side of the chain as in that 4-hydroxyvaleric acid which on reduction gives dextro-methylpropyl carbinol. That acid is dextro-4-hydroxyvaleric acid. Hence in dextro-2-hydroxyvaleric acid the hydroxyl is on the same side of the chain as in dextro-4-hydroxyvaleric acid.

The set of reactions which led to the correlation of dextro-2-hydroxyvaleric acid with levo-methylpropyl carbinol is the following:



For comparison we give the set of reactions leading from dextro-4-hydroxyvaleric acid to dextro-methylpropyl carbinol.



An important practical significance of this conclusion lies in the fact that it furnishes additional evidence in support of the validity of the indirect method for correlating the configurations of substituted aliphatic acids. The dextro-2-hydroxyvaleric acid on passing to its anion changed its rotation to the left, and, according to the rule developed in this laboratory, the acid should belong to the l series, and now it has been shown by the direct method that it does belong to the l series.

EXPERIMENTAL.

Relationship of 2-Hydroxy-n-Valeric Acid to 1,2-Dihydroxypentane.

Dextro-2-Hydroxy-n-Valeric Acid.—The inactive acid was obtained from 2-bromo-*n*-valeric acid. 181 gm. of 2-bromo-*n*-valeric acid were added to 800 cc. of water containing 138 gm. of potassium carbonate. The solution was heated under a reflux condenser on a boiling water bath for 8 hours. It was then cooled, a solution of 50 gm. of concentrated sulfuric acid in 100 cc. of water was added carefully, and the solution was extracted with ether in a continuous ether extractor. The ether extract was dried over sodium sulfate and the ether removed under reduced pressure. The acid was dissolved in warm acetone and neutralized with 1 equivalent of brucine. The brucine salt, which crystallized readily, was recrystallized repeatedly from acetone. It was then decomposed with ammonia in the usual way and the ammonium salt was converted into the barium salt. In water the barium salt had the following rotation.

$$[\alpha]_D^{20} = \frac{-0.31^\circ \times 100}{2 \times 3.16} = -4.9^\circ.$$

The rotation of the free acid was obtained by dissolving 1.000 gm. of the barium salt in slightly more than 1 equivalent of concentrated hydrochloric acid and diluting to 5 cc. with water.

$$[\alpha]_D^{20} = \frac{+0.38^\circ \times 100}{2 \times 12.7} = +1.5^\circ.$$

Levo-Ethyl-2-Hydroxy-n-Valerate.—To 40 gm. of thoroughly dried barium-2-hydroxy-*n*-valerate ($[\alpha]_D^{20} = -4.9^\circ$) suspended in 100 cc. of absolute alcohol a solution of 12 gm. of concentrated sulfuric acid in 50 cc. of absolute alcohol was added slowly. The mixture was vigorously agitated during the addition of the acid solution. It was then refluxed for 5 hours and allowed to stand overnight. The excess acid was neutralized with solid potassium carbonate, dry ether was added, and the solution filtered from salts. After drying over anhydrous sodium sulfate the ether was removed and the ester was distilled under reduced pressure. It distilled at 81°, p = 20 mm. The yield was 18 gm. It analyzed as follows:

7.080 mg. substance: 14.840 mg. CO_2 and 6.165 mg. H_2O.

$C_7H_{14}O_2$.	Calculated.	C 57.49, H 9.65.
	Found.	" 57.15, " 9.74.

In a 1 dm. tube the optical rotation without solvent was $\alpha_D^{20} = -5.05°$.

Levo-1,2-Dihydroxypentane.—This glycol was obtained on reduction of levo-ethyl-2-hydroxy-*n*-valerate ($\alpha_D^{20} = -4.80°$ in a 1 dm. tube) with sodium and glacial acetic acid in the apparatus described by Levene and Allen.[5] The procedure for the reduction was the same as that described previously for the reduction of other hydroxy acids.[6] The glycol distilled at 78–81°, p = 1 mm. It analyzed as follows:

3.742 mg. substance: 8.045 mg. CO_2 and 3.660 mg. H_2O.

$C_5H_{12}O_2$.	Calculated.	C 57.69, H 11.53.
	Found.	" 58.62, " 10.94.

In absolute alcohol it rotated the plane of polarized light as follows:

$$[\alpha]_D^{20} = \frac{-1.53° \times 100}{2 \times 7.80} = -9.8°.$$

Di-(Phenylurethane) of Levo-1,2-Dihydroxypentane.—1 part of levo-1,2-dihydroxypentane ($[\alpha]_D^{20} = -9.8°$) and 2.33 parts of phenylisocyanate were heated on the steam bath for 15 minutes. When the reaction product was allowed to stand overnight, it crystallized. It was recrystallized several times from dilute alcohol. It melted at 110–113° and analyzed as follows:

0.0500 gm. substance: (Kjeldahl) 2.95 cc. 0.1 N acid.

$C_{19}H_{22}O_4N_2$.	Calculated.	N 8.18.
	Found.	" 8.26.

In absolute alcohol it rotated the plane of polarized light as follows:

$$[\alpha]_D^{20} = \frac{-1.33° \times 100}{2 \times 5.44} = -12.2°.$$

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[5] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 433.

[6] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165. Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 593.

Preparation of Dextro-1,2-Dihydroxypentane from d,l-1-Chloro-2-Hydroxypentane.

d,l-1-Chloro-2-Hydroxypentane.—This substance was obtained on condensation of chloroacetaldehyde with propyl magnesium bromide.

A mixture of 150 gm. of chloroacetal and 90 gm. of anhydrous oxalic acid was slowly distilled. The fraction which distilled at 88–110° was collected and diluted with an equal volume of dry ether. The solution was shaken with 80 gm. of powdered calcium chloride for 4 hours. It was then filtered and the filtrate dropped into a well cooled Grignard reagent prepared from 25 gm. of magnesium, 125 gm. of propyl bromide, and 400 cc. of dry ether. The reaction product was allowed to stand overnight and then decomposed with ice and 1:1 hydrochloric acid in the usual manner. The acid solution was extracted with ether several times. The combined ether extracts were washed successively with dilute hydrochloric acid, water, 10 per cent potassium carbonate solution, and water. The ether solution was dried over anhydrous potassium carbonate for 2 hours and then over sodium sulfate overnight. The ether was removed and the remaining product fractionally distilled. A fraction which distilled at 59–62°, p = 14 mm., was collected and analyzed as follows:

3.490 mg. substance: 6.180 mg. CO_2 and 2.800 mg. H_2O.
0.1122 gm. " : 0.1294 gm. AgCl.

$C_5H_{11}OCl$	Calculated.	C 48.94, H 9.06, Cl 28.95.
	Found.	" 48.29, " 8.97, " 28.53.

Chloromethylpropyl Ketone (1-Chloropentanone-(2)).—The chloropentanol obtained as described above was oxidized with potassium dichromate and sulfuric acid. The reaction mixture was steam-distilled and the chloroketone extracted from the distillate with ether. The ether solution was dried over sodium sulfate. After removal of the ether the chloroketone was distilled. It distilled at 55–57°, p = 15 mm. It readily formed a bisulfite compound. It analyzed as follows:

0.1110 gm. substance: 0.1316 gm. AgCl.

C_5H_9OCl	Calculated.	Cl 29.43.
	Found.	" 29.33.

Hydroxymethylpropyl Ketone (Pentanol-(1)-one-(2)).—A mixture of 50 gm. of chloromethylpropyl ketone, 140 gm. of dried potassium

formate, and 100 cc. of dry methyl alcohol was heated under a reflux condenser on a boiling water bath overnight. The reaction mixture was cooled, dry ether was added, and the solution filtered. After drying the solution over sodium sulfate, the solvent was removed and the hydroxyketone was distilled under reduced pressure. The fraction which distilled at 62–64°, p = 18 mm., was collected and employed for reduction to the glycol.

Dextro-1,2-Dihydroxypentane.—To an actively fermenting mixture of 450 gm. of cane sugar, 450 gm. of bakers' yeast, and 4500 cc. of water were added 45 gm. of hydroxymethylpropyl ketone. The reaction mixture was allowed to stand 6 days and then worked up in the usual manner.[7] The glycol thus obtained was redistilled from a flask provided with a Vigreux column. It distilled at 97–99°, p = 13 mm. It analyzed as follows:

5.930 mg. substance: 12.405 mg. CO_2 and 6.110 mg. H_2O.
$C_5H_{12}O_2$. Calculated. C 57.69, H 11.53.
Found. " 57.04, " 11.52.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 2.60° \times 100}{2 \times 8.00} = + 16.2°.$$

Di-(Phenylurethane) of Dextro-1,2-Dihydroxypentane.—The urethane was prepared in the usual way and recrystallized from dilute alcohol. It melted at 107–110°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5.78 cc. 0.1 N acid.
$C_{19}H_{22}O_4N_2$. Calculated. N 8.18.
Found. " 8.09.

In absolute alcohol the optical rotation was

$$[\alpha]_D^{20} = \frac{+ 1.50° \times 100}{2 \times 4.53} = + 16.5°.$$

Conversion of Optically Active 1,2-Dihydroxypentane into Methylpropyl Carbinol.

Dextro-1-Bromo-2-Hydroxypentane.—Into 14.5 gm. of 1,2-dihydroxypentane ($[\alpha]_D^{22} = +16.2°$) cooled in an ice water bath were passed 15

[7] Neuberg, C., and Kerb, E., *Biochem. Z.*, 1918, xcii, 96. Färber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, 1920, cxii, 313.

gm. of dry hydrogen bromide. The reaction mixture was then heated on the steam bath for ½ hour, cooled, ice and chloroform added, and neutralized with solid potassium carbonate. The bromohydrin was extracted with chloroform and the extract dried over sodium sulfate. The chloroform was removed under reduced pressure and the bromohydrin distilled. It boiled at 72–74°, p = 18 mm. It analyzed as follows:

0.1220 gm. substance: 0.1343 gm. AgBr.

$C_5H_{11}OBr$. Calculated. Br. 47.90.
Found. " 46.84.

In a 2 dm. tube without solvent it had the following rotation: $\alpha_D^{20} = +10.5°$.

Dextro-Methylpropyl Carbinol.—The bromohydrin obtained in the above experiment was reduced in alkaline solution with hydrogen in the presence of colloidal palladium. The procedure was the same as that previously described for the reduction of 1-iodo-3-hydroxybutane.[8] The ether extract was dried over sodium sulfate and the ether removed with the aid of a Vigreux column. The carbinol was then distilled and a fraction which distilled at 116–120° was collected. In alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 2.00° \times 100}{2 \times 7.00} = + 14.3°.$$

α-Naphthylurethane of Dextro-Methylpropyl Carbinol.[9]—The urethane was prepared in the usual way. Recrystallized from dilute alcohol, it melted at 94–96°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 4.13 cc. 0.1 N acid.

$C_{16}H_{19}O_2N$. Calculated. N 5.44.
Found. " 5.78.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 1.05° \times 100}{2 \times 2.97} = + 17.7°.$$

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[8] Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxi, 467.

[9] Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 595.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, May, 1928, Vol. lxxvii, No. 2, pp. 671-683.]

LACTONE FORMATION OF CELLOBIONIC AND OF GLUCO-ARABONIC ACIDS AND ITS BEARING ON THE STRUCTURE OF CELLOBIOSE.

BY P. A. LEVENE AND M. L. WOLFROM.*

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 10, 1928.)

A systematic study of the structure of disaccharides was undertaken under the incentive of the St. Andrews School, which, under the leadership of Purdie has developed the method of methylation of polyhydric alcohols and of sugars. Over 25 years have passed since the method was introduced, and even to-day, there is no complete harmony between the views of individual workers on the structure of the common disaccharides.

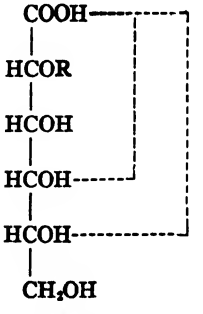
In 1926 Géza Zemplén emphasized the necessity of introducing less drastic methods than those of the St. Andrews School for the study of the structure of disaccharides. Zemplén then suggested a way of progressive degradation of aldobioses until a substance was obtained which no longer was capable of forming an osazone. It was then assumed by him that in the latter substance the hydrogen of the hydroxyl of carbon atom (2) was substituted by the second sugar radicle. The method undoubtedly is very important and ingenious. It has, however, one weak point; namely, that the final conclusion rests on a negative result, which sometimes may be accidental.

A still simpler method was suggested by Levene which is based on the observation of Levene and Simms[1] that each non-substituted sugar acid in solution passes into < 1, 4 > and < 1, 5 > lactones, and that the velocity of formation of each lactone is a function of the ring structure. With this information the structure of a disaccharide can be determined from observations on the mutarotation of the bionic acid obtained on oxidation of the disaccharide and of the bionic

* National Research Fellow in Chemistry.

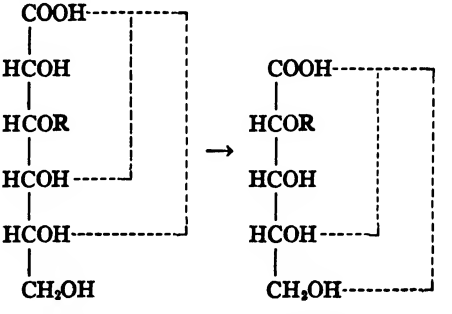
[1] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxv, 31.

acid obtained on removing carbon atom (1) from the original disaccharide. The argument may be best illustrated by the following figures.



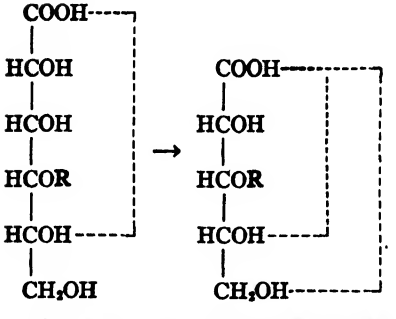
The hexonic acid gives two lactones. It cannot be degraded to a pentose.

I.



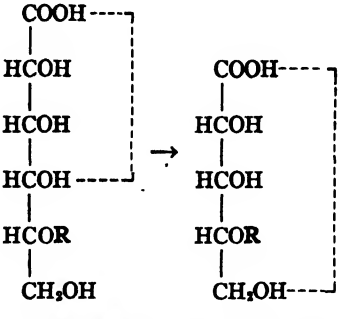
Both the hexonic acid and the corresponding pentonic acid give two lactones.

II.



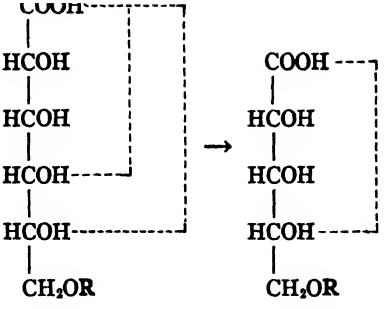
The hexonic acid gives one lactone, < 1, 5 >, and the corresponding pentonic acid two lactones.

III.



The hexonic acid gives one lactone, < 1, 4 >, and the corresponding pentonic acid one lactone, < 1, 5 >.

IV.



The hexonic acid gives two lactones and the corresponding pentonic acid only one, < 1, 4 >.

V.

The work on this plan was initiated by Levene and Sobotka[2] in 1926 and was continued by Levene and Wintersteiner[3] in 1927. The present investigation on the structure of cellobiose was begun in 1927 prior to the publication of the article of Haworth, Long, and Plant.[4] From Tables I to IV and from Figs. 1 and 2 it is seen that cellobionic and the corresponding glucose pentonic acids behave like the substance of formula (III) and that therefore in cellobiose the reducing sugar is substituted in position (4). This conclusion is in harmony with those of Zemplén,[5] Irvine,[6] and Haworth, Long, and Plant.[4]

In the course of the present work an improvement was introduced in the purification of α-cellobiose octaacetate, a procedure was given for obtaining the oxime of cellobiose in crystalline form, an improvement was introduced in the method of the conversion of the oxime into the nitrile octaacetate, a procedure was given for the preparation of the calcium salt of glucoarabonic acid in crystalline form, and also a procedure was developed for the preparation of basic lead acetate suitable for the precipitation of bionic acids and for some monocarboxylic sugar acids.

[2] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926–27, lxxi, 471.

[3] Levene, P. A., and Wintersteiner, O., *J. Biol. Chem.*, 1927, lxxv, 315.

[4] Haworth, W. N., Long, C. W., and Plant, J. H. G., *J. Chem. Soc.*, 1927, cxxxi, 2809.

[5] Zemplén, G., *Ber. chem. Ges.*, 1926, lix, 1254.

[6] Irvine, J. C., *Chem. Rev.*, 1927, iv, 203.

EXPERIMENTAL.

Preparation of Calcium Cellobionate.

Crude α-cellobiose octaacetate, prepared from cellulose, was recrystallized several times from a mixture of 1 part of glacial acetic acid and 5 parts of 96 per cent alcohol. The addition of the acetic acid requires the use of less alcohol and gives about the same yield as when alcohol alone is used. The first recrystallization was easily effected by dissolving the material in 12 parts of the hot solvent mixture instead of the 30 parts required when alcohol alone is used. Further recrystallizations require the higher amount of solvent. The purified material showed a melting point of 222.5° and the following specific rotation in chloroform.

$$[\alpha]_D^{25} = \frac{+ 3.23° \times 100}{2 \times 4.02} = + 40.2°.$$

Hudson and Johnson[7] give the m.p. 229.5° and $[\alpha]_D^{20} = + 41°$ in chloroform for highly purified α-cellobiose octaacetate.

The octaacetate was deacetylated with sodium methylate according to the method of Zemplén[5] and the sugar recrystallized from alcohol and water. This material, dried in a vacuum oven, showed a specific rotation 13 minutes after solution of +22° and a final equilibrium value in water as follows:

$$[\alpha]_D^{25} = \frac{+ 1.55° \times 100}{1 \times 4.43} = + 35.0°.$$

Hudson and Yanovsky[8] give the equilibrium value +35° for pure cellobiose and the value +26° 10 minutes after solution.

Cellobionic acid and a number of its salts were first prepared in amorphous condition by Maquenne and Goodwin.[9] We employed the general method of Willstätter and Schudel as modified by Goebel[10] in oxidizing cellobiose to the bionic acid. 18 gm. (1 mol) of cellobiose were dissolved in 700 cc. of a solution containing 27 gm. (2 mols)

[7] Hudson, C. S., and Johnson, S. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 1276.
[8] Hudson, C. S., and Yanovsky, E., *J. Am. Chem. Soc.*, 1917, xxxix, 1035.
[9] Maquenne, L., and Goodwin, W., *Bull. Soc. chim.*, 1904, xxxi, 854.
[10] Goebel, W. F., *J. Biol. Chem.*, 1927, lxxii, 801.

of iodine and 50 gm. of barium iodide. To this was added over an interval of 5 to 10 minutes, with mechanical stirring, a solution of 50 gm. (3 mols) of barium hydrate in 800 cc. of water. The solution was allowed to stand 15 minutes, after which a solution of 18 cc. of concentrated sulfuric acid in 130 cc. of water was added under strong mechanical stirring. This was followed by the immediate addition of 220 gm. of washed basic lead carbonate. Under continual stirring the solution soon became neutral to Congo red. The precipitate was allowed to settle, filtered on a prepared bed of kieselguhr and charcoal, and the filtrate concentrated under reduced pressure to about 400 cc. The solution was filtered from the separated lead iodide; most of the lead was removed with sulfuric acid and the iodide ion with silver sulfate. After filtration, the remaining lead and silver were removed with hydrogen sulfide, the hydrogen sulfide by air, and the solution again filtered. In the filtrate the excess sulfate ion was removed quantitatively with barium hydroxide and the solution filtered. The filtrate was shaken with an excess of well washed, precipitated calcium carbonate for 15 minutes. Short boiling completed the formation of the calcium salt. The solution was cooled, filtered, and concentrated under reduced pressure to a thin syrup. This was poured into 12 to 15 volumes of anhydrous methyl alcohol. The salt precipitated as a dense, amorphous solid. This was taken up in about 3 times its weight of water and precipitated as a syrup by the addition of $1\frac{1}{2}$ volumes of 96 per cent alcohol. The supernatant liquid was decanted, the residue dissolved in a small amount of water, and poured into 10 to 15 volumes of absolute methyl alcohol. This procedure was repeated. Due to the high solubility of this salt, only 2.5 gm. of purified material were obtained. This, as well as the cadmium and barium salts, resisted repeated attempts at crystallization. The salt analyzed as follows:

6.499 mg. substance: 11.65 mg. $CaSO_4$.

0.1456 gm. dried substance: 0.2028 gm. CO_2 and 0.0720 gm. H_2O. Moisture, 7.30 per cent.

$Ca(C_{12}H_{21}O_{12})_2$.	Calculated.	C 38.18, H 5.61, Ca 5.31.
	Found.	" 37.98, " 5.53, " 5.30.

The salt was kept in a weighed container over calcium chloride and subsequent weighings were corrected for a slow loss of moisture.

Mutarotation and Titration Experiments.

A weight of 0.9020 gm. of calcium cellobionate containing 5.44 per cent of water, thus corresponding to 0.8529 gm. of dry substance,

TABLE I.

Changes in Rotation of 0.090 N Cellobionic Acid Solution.

Experiment 1. l = 2 dm., t = 22°, λ = 5892 Å.			Experiment 2. l = 4 dm., t = 23°, λ = 5892 Å.		
Time.	α	$[\alpha]_D$	Time.	α	$[\alpha]_D$
min.	*degrees*	*degrees*	*min.*	*degrees*	*degrees*
			1	−0.41	−3.2
2	−0.21	−3.3	2	−0.35	−2.7
6	−0.15	−2.3	6	−0.33	−2.5
8	−0.14	−2.2	8	−0.30	−2.3
15	−0.12	−1.9	23	−0.15	−1.2
30	−0.09	−1.4	32	−0.06	−0.5
45	−0.04	−0.6	45	−0.02	−0.2
hrs.			*hrs.*		
1	+0.02	+0.3	1	+0.03	+0.2
2	+0.05	+0.8	2	+0.09	+0.7
4	+0.05	+0.8	4	+0.13	+1.0
6	+0.05	+0.8	6	+0.13	+1.0
20	+0.06	+0.9	24	+0.14	+1.1

TABLE II.

Titration of 1.00 Cc. of 0.090 N Cellobionic Acid Solution with 0.1 N Sodium Hydroxide.

Experiment 1. t = 22°		Experiment 2. t = 23°	
Time.	0.1 N NaOH.	Time.	0.1 N NaOH.
hrs.	*cc.*	*hrs.*	*cc.*
1	0.90	1	0.90
2	0.89		
5	0.89	5	0.90
8	0.90		
20	0.89	23	0.91

was dissolved in about 15 cc. of water. The calculated amount, 4.52 ± 0.01 cc. of 0.5 N hydrochloric acid was added and the solution quickly made up to 25.00 cc. The solution was thus 0.0905 N.

The change in rotation was observed and samples of 1.00 cc., obtained with an accurate micro pipette, were titrated at suitable intervals with 0.1 N sodium hydroxide (phenolphthalein) delivered from a micro burette. Care was exercised that the water used in the experiments did not contain sufficient carbon dioxide to influence the end-point. The data are recorded in Tables I and II and the polarimetric change obtained is plotted in Fig. 1.

The interpolated initial value for the specific rotation of the free acid is $-3.6°$. At the time of the last reading the solution gave no reduction with Fehling's solution, showing that no apparent hydrolysis had taken place.

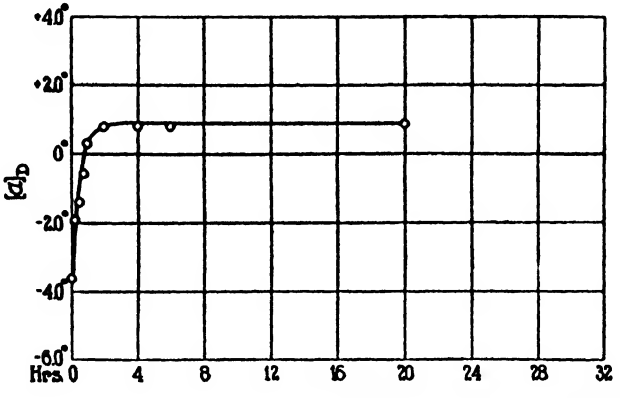


FIG. 1. <1,5> lactone formation of cellobionic acid. Experiment 1.

Preparation of Calcium Glucoarabonate from Cellobiose.

I. Preparation of Crystalline Cellobiose Oxime.—For the preparation of glucoarabinose the procedure of Zemplén[5] was followed with some modification. We were able to prepare cellobiose oxime in crystalline condition. The procedure of Zemplén was followed except that we used pure (99 per cent by Raschig's reduction procedure) hydroxylamine hydrochloride and removed the excess hydroxylamine, after oxime formation, by distillation under reduced pressure, in the presence of solid calcium carbonate to neutralize any acidity. The quantities used by Zemplén in the decomposition of the hydrochloride with sodium methylate leave the former in slight excess. The crude residue was dried by distillation with absolute alcohol. On nucleation the material crystallized. This material was then stirred with

warm 96 per cent alcohol, cooled in an ice-salt mixture, filtered, washed with absolute alcohol, and dried. For the purposes of the next step this material is sufficiently pure. A product more nearly pure was obtained as follows: The crude evaporated product from 43 gm. of cellobiose was dissolved in 3 to 4 times its weight of water, decolorized at room temperature, and filtered. To the filtrate acetone was added with stirring to opalescence. The solution was then nucleated and allowed to stand, first at room temperature and then for some time in the refrigerator. It was filtered, washed with 80 per cent acetone, and then with pure acetone. A weight of 24.2 gm., m.p. 122–123°, was so obtained. The mother liquor was concentrated to 50 cc. and the procedure repeated, 8.2 gm. of product melting at 119–120° being obtained. The filtrate was concentrated to a thin syrup and treated with hot 96 per cent alcohol to slight turbidity, nucleated, and kept overnight in the refrigerator. A further quantity of 1.5 gm. of product melting at 119–120° was obtained, making a total yield of 76 per cent.

Cellobiose oxime crystallizes in plates, is very soluble in water, slightly soluble in cold methyl and ethyl alcohols, moderately soluble in warm methyl and ethyl alcohols, and insoluble in acetone, ether, benzene, ethyl acetate, and petroleum ether (40–60°). On one recrystallization from 80 per cent ethyl alcohol the material melted at 123–125°. A second recrystallization from the same solvent gave material melting at 123–125° and having $[\alpha]_D^{22} = -26.1°$ (1.0313 gm. of substance, 25 cc. of aqueous solution, $\alpha = -2.15°$, 2 dm. tube) in aqueous solution, changing at a very slow rate in the dextro direction. A third recrystallization gave material melting at 123–125° and showing the initial $[\alpha]_D^{24} = -26.1°$ (1.1131 gm. of substance, 25 cc. of aqueous solution, $\alpha = -2.32°$, 2 dm. tube). This purified material analyzed as follows:

5.310 mg. substance: 0.185 cc. N_2 (760 mm., 24°).

$C_{12}H_{23}O_{11}N$. Calculated. N 3.92.
Found. " 4.00.

II. Preparation of Cellobionic Acid Nitrile Octaacetate.—The procedure of Zemplén calls for the acetylation of the syrupy oxime reaction mixture and in our experience always produced a violent

reaction with loss of material and resin formation. With the crystalline oxime this reaction may be easily controlled by the following procedure. A mixture of 140 cc. of acetic anhydride and 21 gm. of fused sodium acetate was heated in an oil bath to 110°. The crystalline oxime (21 gm.) was then added in small portions. Reaction began immediately and the mixture was removed from the oil bath. The addition was continued, the rate being regulated so as to maintain the mixture at 110–115°, and the reaction's own heat being utilized. The addition required about half an hour and was followed by 1 hour of heating at 110°. The preparation was finished according to the directions of Zemplén, the recrystallized product melting at 132° in agreement with Zemplén. It is suggested for similar procedures with other sugars that, where the oxime does not crystallize, it be added gradually to the acetylating mixture in the form of an amorphous powder.

III. Preparation of Calcium Glucoarabonate from Glucoarabinose Heptaacetate.—Glucoarabinose heptaacetate was prepared from cellobionic acid nitrile octaacetate according to the directions of Zemplén. The recrystallized material melted at 194° and showed the following rotation in chloroform.

$$[\alpha]_D^{22} = \frac{-1.20° \times 100}{2 \times 4.42} = -14°.$$

Zemplén records the melting point of 196° and $[\alpha]_D^{22} = -16.95°$ in chloroform solution. 12.5 gm. of the acetylated sugar were deacetylated according to the directions of Zemplén and the resulting solution of sugar and sodium acetate concentrated to 40 cc. to remove methyl alcohol. This solution was oxidized to the aldobionic acid by exactly the same procedure as was previously described for the preparation of cellobionic acid. The amounts of oxidizing reagents used were 19 gm. of iodine, 37 gm. of barium iodide in 500 cc. of water, and 46 gm. of purified barium hydrate in 725 cc. of water, 12 minutes being taken for the addition of the alkali. After removal of the silver and lead ions, the solution containing the oxidation product together with a considerable amount of sodium acetate was neutralized with barium hydroxide solution until it was just acid to litmus. The barium sulfate was removed by filtration and the filtrate concentrated

under reduced pressure at 40° to a volume of 40 cc. A saturated solution of basic lead acetate was added to the solution until no further precipitation occurred. The voluminous precipitate so obtained was filtered and washed with a small amount of very cold water. To the filtrate barium hydroxide was added and a further quantity of precipitate obtained, which was combined with the first.

Fischer and Meyer[11] used basic lead acetate in the preparation of maltobionic acid. They stated that the common form of basic lead acetate did not precipitate the acid and described a method of preparing a basic compound suitable for this purpose. We were unable to obtain active material by repeating their procedure. The right form of basic lead acetate may easily be prepared in crystalline condition by adding barium hydroxide to a solution of ordinary basic lead acetate until a precipitate just begins to form. The solution is then concentrated until a separation of the active basic lead acetate in well formed needles takes place. These are removed by filtration.

The combined precipitates of the basic lead salt of the sugar acid were suspended in about 500 cc. of water and hydrogen sulfide passed into the solution. The solid material was filtered, ground in a mortar, resuspended in the original solution, and again treated with hydrogen sulfide. This process was repeated once. The solution was filtered from the lead sulfide and the hydrogen sulfide removed from the filtrate with a current of air. A trace of barium was removed with sulfuric acid and the most of a small amount of acetic acid present removed by repeated extraction with a considerable volume of chloroform. The filtered solution was shaken with an excess of precipitated and washed calcium carbonate for 15 minutes, boiled 8 minutes, and allowed to stand overnight. After filtration, the solution was concentrated at 40° to a thin syrup and this poured into 10 to 15 volumes of absolute methyl alcohol. The amorphous material was dissolved in water and a mixture of methyl alcohol and acetone added to opalescence. On heating this mixture the material crystallized in radiating clusters of needles. The recrystallization process was repeated, 5 gm. of material being obtained. The analytical results of the material dried first at 100° and then at 110° under reduced

[11] Fischer, E., and Meyer, J., *Ber. chem. Ges.*, 1889, xxii, 1941.

TABLE III.

Changes in Rotation of 0.112 N Glucoarabonic Acid Solution.

l = 4 dm. λ = 5892 Å.

Experiment 1. t = 22°			Experiment 2. t = 23°		
Time.	α	$[\alpha]_D$	Time.	α	$[\alpha]_D$
min.	*degrees*	*degrees*	*min.*	*degrees*	*degrees*
2.5	+2.94	+20.0			
5	2.87	19.5			
7.5	2.84	19.3	6	+2.75	+18.7
10	2.82	19.2	10	2.74	18.6
15	2.78	18.9	15	2.69	18.3
30	2.71	18.4	30	2.62	17.8
45	2.68	18.2	45	2.56	17.4
hrs.			*hrs.*		
1	2.63	17.9	1	2.54	17.3
1.5	2.56	17.4	1.5	2.51	17.1
2	2.51	17.1	2	2.48	16.9
2.5	2.48	16.9			
3	2.50	17.0	3	2.42	16.5
4	2.52	17.1			
5	2.54	17.3	5	2.51	17.1
6	2.57	17.5	7	2.55	17.3
10	2.65	18.0			
23	2.82	19.2	24	2.86	19.4
29	2.91	19.8			

TABLE IV.

Titration of 1.00 Cc. of 0.112 N Glucoarabonic Acid Solution with 0.1 N Sodium Hydroxide.

Experiment 1. t = 22°		Experiment 2. t = 23°	
Time.	0.1 N NaOH.	Time.	0.1 N NaOH.
hrs.	*cc.*	*hrs.*	*cc.*
0.5	1.10		
1	1.10		
2	1.07	1.5	1.10
4	1.05	5.5	1.05
10	1.03		
30	0.89	24	0.86

The permanent titer found by titrating the hot solution was 1.13 cc.

pressure over sulfuric acid indicated that it still contained 1 molecule of water. This behavior is similar to that of the crystalline calcium galactoarabonate obtained by Levene and Wintersteiner.[3]

0.1078 gm. substance: 0.0088 gm. CaO.
0.1046 " " : 0.1424 " CO_2 and 0.0514 gm. H_2O.
$Ca(C_{11}H_{19}O_{11})_2 \cdot H_2O$. Calculated. C 37.06, H 5.66, Ca 5.63.
Found. " 37.12, " 5.49, ". 5.83.

The rotation of the calcium salt was as follows:

$$[\alpha]_D^{28} = \frac{+ 0.27^\circ \times 100}{1 \times 1.88} = + 14.4^\circ.$$

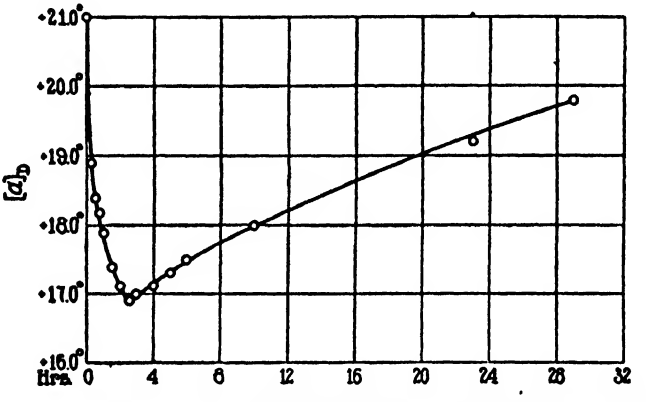


FIG. 2. <1,5> and <1,4> lactone formation of glucoarabonic acid. Experiment 1.

Another preparation gave the following result.

$$[\alpha]_D^{21} = \frac{+ 0.43^\circ \times 100}{1 \times 3.03} = + 14.2^\circ.$$

Mutarotation and Titration Experiments.

A weight of 1.000 gm. of calcium glucoarabonate containing no water other than the molecule of water of crystallization, was dissolved in about 15 cc. of water. The calculated amount, 5.61 ± 0.01 cc., of 0.5 N hydrochloric acid was added and the solution quickly made up to 25.00 cc. The solution was thus 0.1123 N. The change in rotation was observed and samples of 1.00 cc. titrated at suitable intervals with 0.1 N sodium hydroxide (phenolphthalein) as described

with cellobionic acid. The first end-point obtained was recorded. The data obtained are listed in Tables III and IV and the polarimetric change is plotted in Fig. 2.

The interpolated initial value for the specific rotation of the free acid is +21°. At the time of the last reading a sample of the solution containing 30 to 40 mg. of the sugar acid gave no reduction with Fehling's solution, showing that no apparent hydrolysis had taken place.

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STUDIES IN POLYMERIZATION AND CONDENSATION.*

II. PRODUCTS OF INTERREACTION OF POTASSIUM ACETATE AND EPICHLOROHYDRIN.

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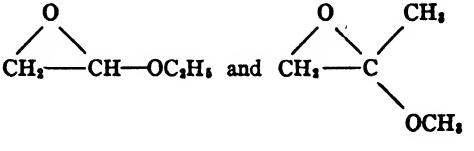
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 30, 1928.)

Many voices have been raised recently against the indiscriminate use of the term "polymerization." The same term is applied to processes of simple aggregation of molecules and to processes of condensation through forces of primary valence.[1]

The confusion in the use of the term is most striking in its application to ethylene oxidic structures. Thus, even in the third edition of Houben's "Die Methoden der organischen Chemie," we find under the same head the process of condensation of ethylene oxide under the influence of zinc chloride or of potassium hydroxide and the spontaneous association of 2 molecules of half acetals of hydroxyaldehydes or of hydroxyketones, such as



In part the confusion is undoubtedly due to the fact that much of the work in this field dates far in the past when the experimental material was not large and when the details of the structures of the so called polymers remained unknown.

The reasons for our undertaking the task of clearing up the

* The first paper of the series is entitled "On Condensation Products of Propylene Oxide and of Glycidol," Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1927, lxxv, 325.

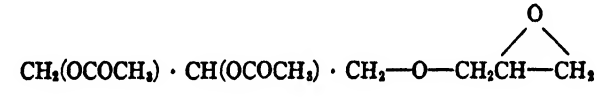
[1] Schroeter, G., *Ber. chem. Ges.*, 1916, xlix, 2697; 1920, liii, 1917. Staudinger, H., *Ber. chem. Ges.*, 1920, liii, 1073. Bergmann, M., *Ann. Chem.*, 1927, cdlii, 121.

structure of the group of ethylene oxidic polymers was given in the first article of this series.[2] In that place were described the condensation products of propylene oxide and of glycidol formed under the simplest conditions.

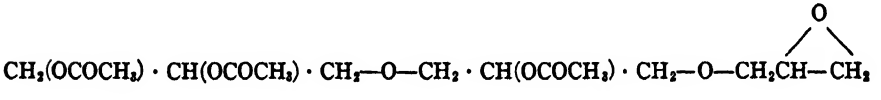
In the present article are described the substances which are formed through the action of dry potassium acetate on epichlorohydrin. The statement was made by Breslauer[3] that under these conditions, in addition to glycidol acetate, a polymer of glycidol was formed. The directions of this author were followed in our experiments.

By means of fractional distillation the following substances were isolated from the reaction product.

1. Acetylglycidol.
2. Diacetin.
3. Diacetylglycerylglycidol.



4. Triacetyldiglycerylglycidol.



5. Higher condensation products.

Each of these products was identified by its carbon and hydrogen content, by its saponification value, and by its molecular weight. The acetyl derivatives were then deacetylated and the free substances were analyzed and then reacetylated.

Thus, under the above mentioned conditions, acetylglycidol has a tendency to condense into di- and polymolecular substances in the same manner as free glycidol.

EXPERIMENTAL.

Experiment 1.

To 200 gm. of finely pulverized dry potassium acetate were added 150 gm. of freshly distilled epichlorohydrin (b.p. 115–116°). This

[2] Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1927, lxxv, 325.

[3] Breslauer, M., *J. prakt. Chem.*, 1879, xx, 188.

mixture was heated for 27 hours, with shaking at intervals, in an oil bath, under a reflux condenser provided with a calcium chloride tube. The temperature was maintained at 120–135° for the greater part of the time, but toward the end of the experiment it was allowed to rise to 150°. After cooling, the reaction product was extracted with dry ether. The ether was removed by distillation and the residue was fractionated into ten fractions.

I. 112–135°, at ordinary pressure; mostly unchanged epichlorohydrin; about 35 gm.
II. 45– 73°, at 25 mm.; mixture of epichlorohydrin and glycidol acetate; 18 gm.
III. 74– 76°, " 25 " glycidol acetate; 35 gm.
IV. 45– 97°, " 0.5 to 1 mm.; 8 gm.
V. 96–100°, " 0.5 mm.; diacetin; 8 gm.
VI. 102–111°, " 0.5 " 2 gm.
VII. 114–125°, " 0.5 " diacetylglycerylglycidol; 6 gm.
VIII. 130–140°, " 0.5 " 2 gm.
IX. 138–155°, " 0.2 " 2 "
X. 153–165°, " 0.2 " 8 "

Fraction V.—This fraction was redistilled and the part boiling at 87–93°, at 0.03 to 0.05 mm., was analyzed. It had the composition of diacetin.

0.0841 gm. substance: 0.1474 gm. CO_2 and 0.0534 gm. H_2O.
$C_7H_{12}O_5$. Calculated. C 47.70, H 6.87.
Found. " 47.79, " 7.12.

Saponification Number.—0.2674 gm. of substance was refluxed with 10 cc. of 0.5 N KOH for 3 hours. 19.1 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 637. Found. 648.

Molecular Weight Determination by Method of Menzies and Wright.[4]—(I) 0.2171 gm. of substance dissolved in 29.7 cc. of benzene (b.p. 80.0°, at 755 mm.) gave an elevation of 27 mm. on the differential thermometer. (II) 0.3493 gm. of substance dissolved in 31.0 cc. of benzene (b.p. 80.5°, at 763 mm.) gave an elevation of 42 mm. on the differential thermometer.

Molecular weight. Calculated. 176. Found. I. 178.
II. 176.

[4] Menzies, A. W. C., and Wright, S. L., Jr., *J. Am. Chem. Soc.*, 1921, xliii, 2314.

Hydrolysis of Fraction V.—This substance was hydrolyzed in the following manner. 6 gm. of the material were refluxed over a free flame with 30 cc. of 5 per cent sulfuric acid. The acetic acid thus formed was extracted by means of ether in a continuous extraction apparatus. The operation was continued 5 hours. From the remaining aqueous solution the sulfuric acid was removed quantitatively by means of a solution of barium hydroxide. The filtrate was then concentrated under reduced pressure and the residue distilled at 130°, at 0.9 mm. Yield 2 gm. The substance had the composition of glycerol.

0.1028 gm. substance: 0.1480 gm. CO_2 and 0.0813 gm. H_2O.
$C_3H_8O_3$. Calculated. C 39.13, H 8.67.
Found. " 39.26, " 8.84.

Acetylation of the Non-Hydrolyzed Substance.—To 3.2 gm. of the above described reaction product (diacetin) were added 10 cc. of acetic anhydride and the mixture was refluxed for $3\frac{1}{2}$ hours. The excess of the anhydride and acetic acid was removed under reduced pressure (10 to 15 mm.). The fraction boiling from 96–98°, at 1.0 mm., was collected. It weighed 1.8 gm. and analyzed for triacetin.

0.0799 gm. substance: 0.1446 gm. CO_2 and 0.0464 gm. H_2O.
$C_3H_5(OCOCH_3)_3$. Calculated. C 49.54, H 6.42.
Found. " 49.35, " 6.49.

Saponification Number.—0.2156 gm. of substance was refluxed for 3 hours with 10.0 cc. of 0.5 N KOH. 21.1 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 772. Found. 752.

Fraction VII.—This fraction (representing diacetylglycerylglycidol) was redistilled and the part boiling at 110–114°, at 0.1 mm., was collected. It analyzed as follows:

4.351 mg. substance: 8.310 mg. CO_2 and 2.785 mg. H_2O.
$C_{10}H_{16}O_6$. Calculated. C 51.73, H 6.89.
Found. " 52.08, " 7.16.

Saponification Number.—0.3139 gm. of substance was refluxed for 3 hours with 15 cc. of 0.5 N KOH and 10 cc. of distilled water. 49.1 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 484. Found. 463.

Molecular Weight Determination by Method of Menzies and Wright.—30.0 cc. of benzene (b.p. 79.8°, at 749 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1113	10	237
0.2431	22	236
0.3836	35	234
0.5633	51	235

Molecular weight calculated, 232.

Hydrolysis of the Substance into Diglycerol.—6 gm. of this substance were refluxed for 6 hours with 30 cc. of 5 per cent sulfuric acid and 10 cc. of water. The solution was worked up as described above. After neutralization with barium hydroxide the filtrate was concentrated and the viscous residue distilled at 196–197°, at 0.5 to 0.6 mm. The yield was 3 gm. of diglycerol. The substance analyzed as follows:

4.589 mg. substance: 7.370 mg. CO_2 and 3.565 mg. H_2O.
$C_6H_{14}O_5$. Calculated. C 43.35, H 8.49.
Found. " 43.79, " 8.69.

Crystalline Diacetylglycerylglycidol from Fraction VII.—On standing for several weeks a crystalline deposit appeared in this fraction. The crystals were filtered off and recrystallized three times from benzene to which a little ligroin was added. After drying at 80° under reduced pressure for 14 hours the substance melted at 125°. It analyzed as follows:

5.940 mg. substance: 11.290 mg. CO_2 and 3.800 mg. H_2O.
$C_{10}H_{16}O_5$. Calculated. C 51.73, H 6.89.
Found. " 51.83, " 7.15.

Saponification Number.—0.0887 gm. of substance was refluxed for 1½ hours with 40 cc. of 0.1 N KOH. 32.4 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 484. Found. 481.

Molecular Weight Determination by Method of Menzies and Wright.—31.5 cc. of benzene (b.p. 80.0°, at 758 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1454	13	229
0.2710	24	231

Molecular weight calculated, 232.

Fraction X.—7.0 gm. of the substance boiling at 153–165°, at 0.2 mm. pressure, were hydrolyzed with 30 cc. of 5 per cent sulfuric acid. 3.0 gm. of diglycerol boiling at 192–193° at 0.2 to 0.3 mm. were obtained. The analysis was as follows:

6.950 mg. substance: 11.400 mg. CO_2 and 5.365 mg. H_2O.
$C_6H_{14}O_5$. Calculated. C 43.35, H 8.49.
Found. " 43.24, " 8.63.

Experiment 2.

In this experiment the time of reaction was extended to 48 hours. To 450 gm. of powdered dry potassium acetate were added 370 gm. of freshly distilled epichlorohydrin. The mixture was heated at 125–130° for 24 hours, at 130–140° for 12 hours, and at 140–155° for 12 hours, and then extracted with ether. The ether was removed by distillation and from the residue the following fractions were obtained.

I. 110–120°, at ordinary pressure; unchanged epichlorohydrin; 70 gm.
II. 70– 80°, " 21 mm.; crude glycidol acetate; 40 gm.
III. 35– 95°, " 1 " 6 gm.
IV. 95–110°, " 0.7 " 6 "
V. 108–130°, " 0.8 " 35 "
VI. 128–155°, " 0.3 " 25 "

At this point the distillation was interrupted. After 2 hours it was resumed. The part which came over at 90°, at 0.3 mm. pressure, contained platelets which continuously increased in size until some of the crystals reached the length of 1 inch. They were arranged in rosettes in the upper and side parts of the receiver. About 12 gm. of substance came over before the temperature rose to 165°, at 0.3 mm. (Fraction VII). The final fraction boiled at 165–210°, at 0.2 to 0.5 mm. (Fraction VIII). Some decomposition took place at this temperature.

The residue weighed about 120 gm. It was dark in color and reacted neutral to litmus paper, was soluble in benzene, acetone, and alcohol and insoluble in ether and water. Inasmuch as the original material was soluble in ether, it is possible that this product was formed during distillation, although the possibility is not excluded that the product was soluble in ether in the presence of the material which came over in the other fractions.

Fraction Boiling from 128–155° (Fraction VI).—This portion was redistilled with the aid of a small column. From it the following fractions were obtained.

IX. 104–114°, at 0.2 mm.; 5.3 gm.
X. 112–114°, " 0.1 " 6.6 " diacetylglycerylglycidol.
XI. 130–140°, " 0.1 " 3.6 "
XII. 140–155°, " 0.1 " 3.6 "

To the residue was added the portion boiling at 0.2 mm. from 153–165° (Fraction X of Experiment 1). It was then distilled below 100°, at 0.1 mm. 3.5 gm. were collected and at the same time 0.4 gm. of crystals separated. On continuing the distillation the following fractions were obtained.

XIII. 155–168°, at 0.05 to 0.03 mm.; 5.8 gm.
XIV. 200–210°, " 0.3 " 0.6 " 3.7 "

Acetylation of Diglycerol.—All fractions boiling between 112–160°, at 0.03 to 0.3 mm., *i.e.* Fractions VII, VIII, IX, and X of Experiment 1 as well as Fractions X, XI, and XII of Experiment 2, yielded diglycerol on acid hydrolysis. There was an indication of the presence of a small quantity of glycerol also. The carbon content of the higher boiling fractions (130–210°, at 0.2 mm. or less) varied little (50 to 51 per cent).

To 3 gm. of the diglycerol obtained from one of these experiments were added 15 cc. of redistilled acetic anhydride and the mixture was refluxed for 3 hours. The excess of the anhydride was removed under diminished pressure with the aid of a small fractional distilling flask. The fraction boiling from 140–153°, at 0.05 to 0.1 mm., was collected. It analyzed for tetraacetyldiglycerol.

5.534 mg. substance: 10.253 mg. CO_2 and 3.405 mg. H_2O.
$C_{14}H_{22}O_9$. Calculated. C 50.28, H 6.64.
Found. " 50.52, " 6.88.

Saponification Number.—0.2841 gm. of substance was refluxed for 3 hours with 10 cc. of 0.5 N KOH. 16.25 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 671. Found. 666.

Molecular Weight Determination by Method of Menzies and Wright.—30.1 cc. of benzene (b.p. 80.1°, at 758 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1576	10	338
0.3597	23	336
0.5507	35	338
0.9522	59	346

Molecular weight calculated, 334.

Fraction XIII.—The fraction boiling from 155–168°, at 0.05 to 0.03 mm., was very probably a mixture of a larger amount of triacetyldiglycerol and an acetylated triglycerol derivative. It analyzed as follows:

0.0885 gm. substance: 0.1622 gm. CO_2 and 0.0542 gm. H_2O.

$C_{12}H_{20}O_8$ (Triacetyldiglycerol). Calculated. C 49.30, H 6.55.
Found. " 49.97, " 6.85.

Saponification Number.—0.2566 gm. of substance was refluxed for 2 hours with 10.5 cc. of 0.5 N. KOH. 26.5 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated for triacetyldiglycerol. 576.
Found. 568.

Molecular Weight Determination by Method of Menzies and Wright.—30.0 cc. of benzene (b.p. 80.6°, at 769 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.2843	22	283
0.4620	33	307
0.6402	44	316

Molecular weight calculated for triacetyldiglycerol, 292.

Hydrolysis of Fraction XIII.—3.8 gm. of substance were heated gently for 12 hours with 20 cc. of 5 per cent sulfuric acid. The reaction product was freed from acetic and sulfuric acids and concentrated. The anhydrous residue weighed 1.9 gm. On distillation about 0.2 gm. came over at the boiling point of glycerol while the main fraction distilled at 176–180°, at 0.06 mm. The analysis was as follows:

0.1185 gm. substance: 0.1914 gm. CO_2 and 0.0896 gm. H_2O.
$C_6H_{14}O_5$ (Diglycerol). Calculated. C 43.35, H 8.49.
Found. " 44.04, " 8.46.

This would indicate that this fraction represents a mixture of di- and triglycerol.

Fraction XIV.—The fraction boiling at 200–210°, at 0.3 to 0.6 mm., weighed 3.7 gm. and analyzed as follows:

0.1068 gm. substance: 0.1996 gm. CO_2 and 0.0674 gm. H_2O.
Found. C 50.96, H 7.06.

Saponification Number.— 0.2109 gm. of substance was refluxed for 2 hours with 10 cc. of 0.5 N KOH. 31.3 cc. of 0.1 N HCl were required for neutralization. Saponification number found, 499.

Hydrolysis of Fraction XIV.—This substance was hydrolyzed with 20 cc. of 5 per cent sulfuric acid as described above. During this operation the odor of acrolein was perceived, indicating that the material contained some decomposition products of glycerol which had been formed by the high temperature of the distillation. The acetic and sulfuric acids were removed in the manner described above and the remaining solution was concentrated. The anhydrous residue was fractionated by distillation. A trace of substance came over at the boiling point of glycerol, then a small amount between 200–205°, at 0.1 mm. *This fraction analyzed for triglycerol.*

0.1072 gm. substance: 0.1852 gm. CO_2 and 0.0808 gm. H_2O.
$C_9H_{20}O_7$. Calculated. C 45.00, H 8.40.
Found. " 45.34, " 8.50.

Distillation Residue.—This material was a dark viscous mass which had a strong odor of acrolein, thus showing that some decomposition took place during distillation. The composition of the substance was the following.

0.1094 gm. substance: 0.2100 gm. CO_2 and 0.0678 gm. H_2O.
Found. C 52.34, H 6.93.

Saponification Number.—0.5522 gm. of substance was refluxed with 12 cc. of 0.5 N KOH for 3 hours. 19.9 cc. of 0.1 N HCl were required for neutralization. Saponification number found, 407.

Molecular Weight Determination by Method of Menzies and Wright.—30.7 cc. of benzene (b.p. 80.2°, at 760 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.5181	10	1095
1.0354	19	1150
1.7045	30	1200
2.3702	39	1280
2.8913	48	1275
3.1132	52	1265

Acetylation of Material.—10 gm. of this residue and 28 gm. of freshly distilled acetic anhydride were refluxed for 3 hours. The low boiling material was removed by distillation, first at reduced pressure (about 10 to 15 mm.) and subsequently at a pressure of 0.5 mm. at 230°. The residue analyzed as follows:

0.0988 gm. substance: 0.1908 gm. CO_2 and 0.0592 gm. H_2O.
Found. C 52.66, H 6.70.

Saponification Number.—0.2976 gm. of substance was refluxed with 10.1 cc. of 0.5 N KOH for 3 hours. 24.4 cc. of 0.1 N HCl were required for neutralization. Saponification number found, 493.

Molecular Weight Determination by Method of Menzies and Wright.—30.8 cc. of benzene (b.p. 79.6°, at 745 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.5214	10	1075
0.8218	16	1060
1.2828	23	1150
1.9014	36	1090
2.8051	53	1090
3.2452	61	1095

Hydrolysis of Residue.—To 31 gm. of the original residue were added 100 cc. of 5 per cent sulfuric acid and the solution was refluxed for 6

hours. The acetic and sulfuric acids were removed by the procedure described above. The hydrolyzed product could not be distilled at 0.5 mm. pressure although the outside temperature was raised to 230°. At this temperature the substance began to decompose. From the analysis and from the high boiling point of this substance, and from the molecular weight determination of the acetate, it may be concluded that *the product is made up of a long chain of glyceryl rests, perhaps not less than seven.* The analysis of the hydrolysate was as follows:

0.0903 gm. substance: 0.1585 gm. CO_2 and 0.0636 gm. H_2O.
$C_{21}H_{44}O_{16}$. Calculated. C 47.00, H 8.27.
Found. " 47.86, " 7.88.

Acetylation of This Hydrolyzed Residue.—To 10 gm. of the hydrolyzed residue were added 28 gm. of freshly distilled acetic anhydride and the mixture was refluxed for 3 hours. The product, which was worked up as described above, could not be distilled at 230°, at 0.2 to 0.4 mm. The analysis was as follows:

0.1003 gm. substance: 0.1887 gm. CO_2 and 0.0626 gm. H_2O.
$C_{39}H_{52}O_{24}$ (Nonoacetylhexaglycerylglycerol). Calculated. C 51.19, H 6.83.
Found. " 51.30, " 6.98.

Saponification Number.—0.3534 gm. of substance was refluxed with 10.05 cc. of 0.5 N KOH for 3 hours. 17.90 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 552. Found. 512.

Molecular Weight Determination by Method of Menzies and Wright.—29.8 cc. of benzene (b.p. 80.2° at 760 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
1.3126	30	952
1.6647	38	953
2.1109	49	937
2.3965	56	931
2.8474	67	925

Molecular weight calculated, 915.

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STUDIES IN POLYMERIZATION AND CONDENSATION.

III. ON AUTOCONDENSATION OF DIHYDROXYACETONE.

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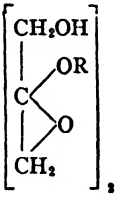
The present work was undertaken because of our interest in the natural tendency of molecules with an ethylene oxidic group to form complexes of high molecular weights and because of our interest in the molecular structures of the substances formed in this manner. Dihydroxyacetone is supposed to crystallize in a dimeric form. To the dimeric half acetals Fischer and his coworkers[1] assign the following ethylene oxidic structure.



Thus dihydroxyacetone is assumed to be capable of combining by molecular forces into polymeric forms. On the other hand, it should be capable of condensing with itself through loss of water into half acetals. The important question then arose as to which of the two reactions predominates when dihydroxyacetone is allowed to stand at ordinary temperature in the absence of other catalytic agents save the oxygen of the air. The immediate occasion for the present study was the following.

Dr. Herzog of H. A. Metz Laboratories, Inc., generously placed at our disposal a quantity of oxanthase which had been kept in their storeroom for about a year and which had turned into a sticky mass containing a considerable quantity of crystals. Thus the experi-

[1] Fischer, H. O. L., and Mildbrand, H., *Ber. chem. Ges.*, 1924, lvii, 707. Fischer, H. O. L., and Taube, C., *Ber. chem. Ges.*, 1924, lvii, 1502.

ment which we had intended to perform had already taken place and it remained for us to analyze the products formed in the course of this experiment.

Pure dihydroxyacetone was prepared first by Piloty[2] in 1897. From an acetone solution of this substance a crystalline product formed. The observations of Piloty on the conduct of his substance are very significant. The substance formed on the first crystallization and regarded as dihydroxyacetone melted at 68–75°. This indefinite melting point was explained by the assumption of polymerization during the process of melting. The significant point noted in this connection by Piloty was that by repeated recrystallization the melting point of the substance did not become sharper, but on the contrary, become more protracted, the point of complete melting continually rising. Later in his article Piloty describes in greater detail the conduct of dihydroxyacetone on standing. When liquid dihydroxyacetone was allowed to stand without cooling and without seeding with the crystalline material to induce crystallization, it was transformed after a long interval into a crystalline substance which was not dihydroxyacetone but a polymerization or condensation product of it, melting at 155°. When dihydroxyacetone was heated to 60–70°, under reduced pressure, an amorphous substance was obtained which dissolved in water very slowly, but not at all in alcohol. On boiling with dilute mineral acid it dissolved in a cloudy solution which reduced Fehling's solution in the cold. Piloty remarked that the new products were either polymerization or condensation products, but lack of material did not permit him to study the details of their structure.

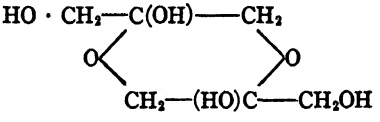
The very important work on dihydroxyacetone which has been done in more recent years was not concerned with the significant findings of Piloty. Bertrand[3] developed a practical method for the preparation of dihydroxyacetone by means of *Bacterium xylinum*. Bertrand made molecular weight estimations of the crystalline dihydroxyacetone by the cryoscopic method and assigned to it a dimeric structure, assuming that the monomeric form was a syrup.

[2] Piloty, O., *Ber. chem. Ges.*, 1897, xxx, 3161.

[3] Bertrand, G., *Compt. rend. Acad.*, 1898, cxxvi, 842, 984; *Ann. chim. et physique*, 1904, iii, series 8, 215, 246.

The crystalline form according to Bertrand melts at about 80° (not sharp).

Wohl and Neuberg[4] suggested for the dimeric form the half acetal structure



Finally Fischer[1] and his coworkers made the important discovery that the substance of Bertrand on distillation at 0.4 to 0.6 mm. pressure at the temperature of the bath at 125–130° yields a syrup which crystallizes in the monomeric form, melting between 65–71°.

From this brief survey it is seen that the very important observations of Piloty have not received the attention which they deserve in the light of the modern speculations regarding the structure of the natural products of high molecular weight.

The material placed at our disposal was a product very similar to the one described by Piloty. Possibly the material had had a chance to undergo further changes than that of Piloty, inasmuch as it had aged for a longer period of time. The sticky material on drying to constant weight at about 75° and reduced pressure contained 42.7 per cent carbon and 5.95 per cent hydrogen. Cryoscopically determined, the molecular weight was about 200, which corresponds to the dimolecular form. It reduced Fehling's solution in the cold. It had no sharp melting point, liquefying at 120°. Its reducing power was about 40 per cent of that of glucose. It gave a triose phenylosazone and a bisulfite derivative. The crude product was fractionated into three parts by means of neutral solvents only, all operations being conducted at room temperature.

Extract I.—Extract obtained from the crude material by extraction with a solution consisting of equal parts of 98.5 per cent alcohol and anhydrous ether.

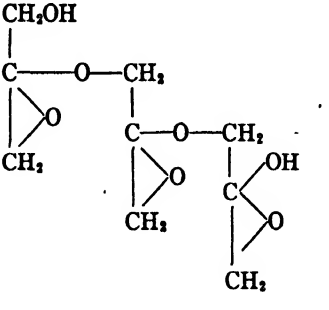
Extract II.—Extract obtained from the first residue by extraction with acetone.

Residue II.—Final residue.

[4] Wohl, A., and Neuberg, C., *Ber. chem. Ges.*, 1900, xxxiii, 3095.

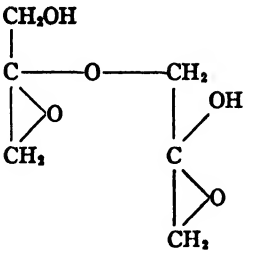
From Extract I were isolated the following substances: volatile fatty acids, the nature of which has not been studied, methylglyoxal, monomeric dihydroxyacetone, and higher condensation products of dihydroxyacetone, the structure of which has not yet been studied in detail. The peculiarity of the monomeric dihydroxyacetone which was obtained by us lay in its melting point which was found quite sharp at 82°. Thus the indefinite melting points reported by previous observers were most likely due to impurities and not to polymerization during the process of melting.

From Extract II also the monomeric form was obtained by distillation at 0.2 to 0.4 mm. pressure and a bath temperature of 120–125°. The residue from the distillation had a sharp melting point at 258°. It was insoluble in water and practically insoluble in all organic solvents, but was soluble in dilute aqueous alkali. It reduced Fehling's solution in the cold; the reducing power in the cold being 11.1 per cent and on warming 22.7 per cent of that of glucose. It did not form an insoluble phenylosazone nor a bisulfite derivative. It had the composition of $C_9H_{14}O_7$, and presumably had the following structure.



Whether this substance was preformed or was developed during the process of distillation is difficult for the present to state with certainty.

From Residue II a crystalline substance was obtained which had a sharp melting point at 164°. It was soluble in cold water, in hot methyl, ethyl, and butyl alcohols and very readily soluble in pyridine. It reduced Fehling's solution in the cold. The substance analyzed exactly for $C_6H_{10}O_5$. This substance then has the following structure.



All reactions leading to this substance were conducted at room temperature, and hence the conclusion is warranted that *on standing, dihydroxyacetone spontaneously condenses through loss of water into substances of higher molecular weight. Polymerization of Bertrand's substance into true polymers of higher molecular weight has not been observed.*

In fact, the present investigation points to the necessity of reinvestigating the nature of Bertrand's substance. Is it actually a dimeric form or is it a mixture of the monomeric form contaminated with some of the condensation products? Indeed, it is not at all clear why the monomeric form isolated by us should have the same melting point as the dimeric form of Bertrand. On the other hand, should the generally accepted view of the molecular state of Bertrand's substance be substantiated, and thus should it be proved that the parent substance of the condensation products is the dimeric dihydroxyacetone, then *the conclusion will be warranted that polymerization is the initial phase in the process of condensation. The true polymers, then, are groupings analogous to Kekulé's giant molecules.*

EXPERIMENTAL.

A. Properties of the Crude Material.

The crude material consisted of crystals imbedded in a thick syrup, the material as a whole having the character of a sticky mass. It was soluble in hot alcohol and in water. The aqueous solution reacted acid to litmus. It reduced Fehling's solution in the cold. The reducing power by the method of Maquenne and Lehmann was 40 per cent of that of glucose. With phenylhydrazine it gave an osazone in the cold.

The composition of the crude mass dried to constant weight under reduced pressure at the temperature of chloroform vapor was as follows:

4.905 mg. substance: 7.680 mg. CO_2 and 2.620 mg. H_2O.
Found. C 42.69, H 5.95.

The molecular weight determined cryoscopically in water was the following.

13.0000 gm. H_2O, 0.2948 gm. substance; 0.210° freezing point depression.
Found, molecular weight 201.
13.0000 gm. H_2O, 0.5540 gm. substance; 0.410° freezing point depression.
Found, molecular weight 197.

B. Fractionation of the Crude Material.

1. Extract I.—The original sticky mass (about 500 gm.) was suspended in 2 liters of a solution consisting of equal parts by volume of alcohol (98.5 per cent) and of anhydrous ether. The supernatant liquid was decanted and the operation was repeated until the remaining residue acquired the character of a slightly soft solid. About ten extractions were required.

(*a*) *Properties of Extract I.*—All extracts were filtered and concentrated under reduced pressure to a thick syrup. This syrup was soluble in water, and in alcohol. An aqueous solution reacted acid to litmus. The aqueous solution formed a phenylosazone in the cold. It formed a bisulfite derivative. The reducing power in the cold of the syrup was 25 per cent of that of glucose.

(*b*) *Fractionation of Extract I.*—The distillation was carried out in the following way. 40.0 gm. of the syrup, which still contained about 20 per cent of alcohol, were introduced into a Claisen flask which had the side tube bent downward amost vertically in a manner to permit convenient insulation of the distilling system by means of asbestos paper and thus to avoid clogging the side tube by the crystals forming on cooling the distillate. The vapors were allowed to pass two receivers, the first consisting of an ordinary distilling flask cooled with running water. This flask in its turn was connected with a U-tube which was cooled by a mixture of solid carbon dioxide and acetone.

The distillation was at first conducted without special precaution until the temperature of the bath reached 70°, at 15 mm. pressure. In this manner all the residual alcohol as well as some volatile acids and some methylglyoxal were removed. When this temperature was reached the above described system was introduced and the distillation then proceeded at very low pressures. Three fractions were collected at 0.2 to 0.6 mm. pressure and at the temperature of the vapors from 93–110° (120–130° of the bath). The more volatile material collected in the U-tube, the less volatile in the first receiver, and finally the residue in the original distilling flask.

(*c*) *The More Volatile Fraction of Extract I.*—This portion consisted chiefly of volatile fatty acids, perhaps principally of formic acid and of methylglyoxal. The acids have not been identified. The latter was identified as its phenylosazone which was prepared in the usual way. It was recrystallized from hot alcohol and washed with cold alcohol repeatedly. It then melted at 145° and had the following composition.

3.365 mg. substance: 0.645 cc. N_2 at 764 mm. and 24°.

$C_{18}H_{16}N_4$.	Calculated.	N 22.21.
	Found.	" 22.23.

The distillate gave with Schiff's reagent a red coloration on standing and when heated to boiling it developed yellowish green vapors which are characteristic of methylglyoxal.

(*d*) *The Less Volatile Fraction of Extract I.*—The distillate collected in the first receiver consisted of a thick syrup filled with a mass of crystals. The mass was extracted in the cold first by a solution of alcohol and ether (1:1) and then by acetone.

The crystals had a very sharp melting point at 82°. The reducing power toward Fehling's solution was about 60 per cent of that of glucose. The substance formed a phenylosazone in the cold and readily formed a bisulfite derivative. It had the elementary composition of $C_3H_6O_3$ as is seen from the following data. The molecular weight determined cryoscopically in water showed its monomeric composition.

15.1584 gm. H_2O, 0.2907 gm. substance; 0.407° freezing point depression.
Found, molecular weight 87.5.

15.1584 gm. H_2O, 0.5047 gm. substance; 0.707° freezing point depression.
Found, molecular weight 87.5; calculated, 90.0.

The substance had the following composition.

4.675 mg. substance: 6.920 mg. CO_2 and 2.860 mg. H_2O.
$C_3H_6O_3$. Calculated. C 40.00, H 6.66.
Found. " 40.36, " 6.84.

(*e*) *Distillation Residue (18.5 Gm.) of Extract I.*—This was apparently a very complex mixture. *En masse*, it analyzed for $C_6H_{10}O_5$, that is, for a substance formed from 2 molecules of dihydroxyacetone through loss of water, as seen from the following analytical data.

0.1030 gm. substance: 0.1700 gm. CO_2 and 0.0566 gm. H_2O.
$C_6H_{10}O_5$. Calculated. C 44.42, H 6.17.
Found. " 45.00, " 6.14.

In fact, it was easily proved that the material was a mixture of condensation products with the monomeric dihydroxyacetone or possibly with a true dimeric form. The hydroxyacetone tenaciously adhered to the condensation product.

The product was acetylated in the following way. To 5 gm. of the brown-colored residue were added 30 cc. of acetic anhydride and the mixture was refluxed for 2 hours. The excess of anhydride was removed by distillation at 15 mm. pressure. The distillation was then continued at 0.1 to 0.2 mm. pressure. At 85° 1.4 gm. of crude diacetyldihydroxyacetone distilled over. The distillate crystallized in the receiving flask in long needles. The crystalline material was recrystallized from ether and petrolic ether and the crystals were then dried on hardened filter paper. The substance had the same melting point as recorded by Dimroth and Schweizer,[5] namely 46–47°. It analyzed as follows:

5.815 mg. substance: 10.320 mg. CO_2 and 2.965 mg. H_2O.
$C_7H_{10}O_5$. Calculated. C 48.28, H 5.75.
Found. " 48.39, " 5.70.

The distillation residue showed the composition of a condensation product.

4.645 mg. substance: 8.670 mg. CO_2 and 2.325 mg. H_2O.
Found. C 50.9, H 5.6.

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[5] Dimroth, O., and Schweizer, R., *Ber. chem. Ges.*, 1923, lvi, 1375.

(*f*) *Fractionation after Preliminary Extraction with Ether.*—A portion of the concentrate of Extract I was dissolved in very little water and extracted with ether for 30 hours. The residue was concentrated under reduced pressure to remove the water and then distilled at 0.2 to 0.5 mm. pressure. At the temperature of the bath of about 120–125°, a distillate came over consisting of the monomeric dihydroxyacetone. In this experiment the residue did not have the dark brown color which it did in the other experiment, but was only slightly yellow. It had a waxy appearance. This material was acetylated and the acetylation product was distilled. At a pressure of 0.2 to 0.5 mm. and at a temperature of the bath of 110–125°, a fraction came over which had the melting point of diacetyldihydroxyacetone. At the temperature of the vapors of 138–150°, at 0.5 to 0.8 mm. pressure, an oil came over which had the composition of diacetyldihydroxyacetonyldihydroxyacetone. The substance gave the following analysis.

3.490 mg. substance: 6.230 mg. CO_2 and 1.835 mg. H_2O.
$C_{10}H_{14}O_7$. Calculated. C 48.76, H 5.73.
Found. " 48.68, " 5.88

The molecular weight in benzene by the method of Menzies and Wright was as follows: 32.0 cc. of benzene (b.p. 80.4° at 767 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1294	9.2	287
0.2949	20.8	289

$C_{10}H_{14}O_7$. Molecular weight calculated, 246.

2. Residue I.—This residue is the material remaining after extraction of the crude material with alcohol and ether. As stated above, it had the character of a slightly soft solid. The reducing power of the substance toward Fehling's solution in the cold was equivalent to 40 per cent of that of glucose. The material treated with phenylhydrazine in the cold formed an osazone nearly instantaneously, but the quantity of osazone formed continually increased. For analysis it was recrystallized from alcohol. Its composition was as follows:

3.240 mg. substance: 0.596 cc. N_2 at 764 mm. and 25°.
4.465 " " : 11.075 mg. CO_2 and 2.300 mg. H_2O.
$C_{18}H_{16}ON_4$. Calculated. C 67.13, H 6.00, N 20.88.
Found. " 67.64, " 5.76, " 21.10.

3. Extract II.—Residue I was covered with acetone and thoroughly ground in a mortar, the acetone being renewed until the soft solid turned into a dry powder (Residue II). The extracts were combined, filtered, and concentrated under reduced pressure at room temperature. A thick syrup was obtained in this manner much more viscous than Extract I. This syrup was then distilled at 0.2 to 0.6 mm. pressure and at the temperature of 93–110°. The distillate was treated exactly as that in the case of Extract I. From it monomolecular dihydroxyacetone was obtained, with the same properties as the corresponding fraction of Extract I. The residue had a different appearance from that of the corresponding fraction of Extract I. It was lighter in color and had a harder consistency. After a preliminary test it was found most convenient to take the mass up in warm dilute methyl alcohol. The greatest part dissolved, leaving a certain amount of undissolved crystals. To this suspension an equal volume of acetone was added and the suspension was allowed to stand overnight and filtered. This substance was found insoluble in water, in alcohol, in acetone, in pyridine, in glacial acetic acid, and in acetic anhydride. It was soluble in dilute aqueous alkali. The melting was sharp at 252°. The reducing power for Fehling's solution was 11.10 per cent of that of glucose in the cold and 22.72 per cent on warming. Its composition was C 45.5, H 5.91. For further purification the crystalline material was exhaustively extracted with acetone. Through this purification the melting point was raised to 258°. The solubility was not altered, nor was the reducing power. The composition of the substance was the following.

6.360 mg. substance: 10.750 mg. CO_2 and 3.385 mg. H_2O.
$C_9H_{14}O_7$. Calculated. C 46.15, H 5.98.
Found. " 46.09, " 5.95.

Thus the substance seems to be composed of 3 molecules of dihydroxyacetone with the loss of 2 molecules of water.

4. Residue II.—This is the residue obtained from the crude material by extraction with a solution of equal parts of alcohol and ether, and subsequently with acetone. It was a dry powder with the same solubilities and the same reducing properties as Residue I. How-

ever, it no longer formed an insoluble osazone as Residue I did, perhaps owing to the fact that it contained only a little of the uncondensed dihydroxyacetone. This residue was soluble in hot methyl, ethyl, and butyl alcohols and in pyridine at room temperature. The substance contained 42.83 per cent of carbon and 5.93 per cent of hydrogen.

3.0 gm. of this substance were dissolved in pyridine, 2 volumes of ether were added, and the solution concentrated, under reduced pressure (12 mm.), nearly to dryness. The residue consisted of a syrupy mass imbedded with crystals. The crystals were freed from the mother liquor by means of a solution of equal parts of alcohol and ether. The crystalline material obtained in this manner melted sharply at 164°. The reducing power was 75 per cent of that of glucose. It did not form an insoluble phenylosazone, nor did it form a bisulfite derivative. The substance analyzed correctly for $C_6H_{10}O_5$ as seen from the following data.

5.680 mg. substance: 9.240 mg. CO_2 and 3.340 mg. H_2O.

$C_6H_{10}O_5$. Calculated. C 44.42, H 6.17.
Found. " 44.36, " 6.59.

When the experiment was performed on a larger scale a substance was obtained of lesser purity having only 43.8 per cent of carbon. Some of this material was acetylated as follows: To 1.5 gm. were added 8 cc. of freshly distilled acetic anhydride, the mixture was refluxed for 1 hour, and the excess of anhydride was removed by distillation at 15 mm. pressure. The acetylation product was distilled at 0.1 to 0.2 mm. pressure and at a temperature of 83–85°. The distillate consisted of diacetyldihydroxyacetone which crystallized on cooling. The crystals had a melting point of 46–48°. The residue consisted of diacetyldihydroxyacetonyldihydroxyacetone as seen from the following analytical data.

7.270 mg. substance: 13.120 mg. CO_2 and 3.750 mg. H_2O.

$C_{10}H_{14}O_7$. Calculated. C 48.76, H 5.73.
Found. " 49.21, " 5.77.

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ON WALDEN INVERSION.

XII. ON THE OXIDATION OF 3-THIOLVALERIC AND OF 4-THIOLVALERIC ACIDS AND ITS SIGNIFICANCE IN CONNECTION WITH WALDEN INVERSION.

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In previous investigations of Levene and Mikeska[1] and of Levene, Mori, and Mikeska[2] a series of thiolcarboxylic acids has been compared with the corresponding sulfo acids with respect to their optical behavior. It was found that the undissociated acids on passing into the ionized state showed a change in rotation the direction of which was identical in the thiol and in the sulfo acids. On the basis of these observations the conclusion was formulated that the change of polarity of the substituting group does not alter the direction of rotation which the original acid displayed on passing from the unionized to the ionized state. In its turn, this conclusion furnished a way of recognizing those reactions of substitution in the simple aliphatic acids which are accompanied by a Walden inversion.

In the series of thiol- and sulfocarboxylic acids previously analyzed, only one exception was encountered. Namely, levo-3-thiolbutyric acid on passing to the mono-ion and then to the di-ion showed a change of rotation towards the right, whereas the sulfo acid derived from it on passing from the undissociated state to the mono- and then to the di-ion changed its rotation to the left. The question naturally arose as to the cause of this exceptional behavior.

The difference between the structure of the 3-thiolbutyric acid and the other acids which had been analyzed rests principally in the distance of the thiol group from the carboxyl. Whereas all the other

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[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1926, lxx, 365.

[2] Levene, P. A., Mori, T., and Mikeska, L. A., *J. Biol. Chem.*, 1927, lxxv, 337.

acids were substituted in position (2), this one had the reactive group on carbon atom (3).

Hence, it was necessary to investigate the behavior of a larger group of acids substituted on carbon atoms (3) or (4) and possibly still more distantly from the carboxyl.

The present communication contains a report on the valeric acids substituted in positions (3) or (4).

The configurational relationships of 3- and 4-hydroxyvaleric acids to lactic acid have been established by Levene and Haller.[3] In Table I are given the rotations of the undissociated acids and of the

TABLE I.

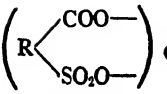
Molecular Rotations of Substituted Valeric Acids.

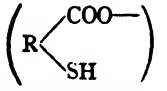
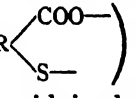
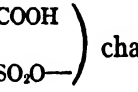
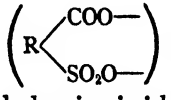
	Hydroxy.	Halide.	Thiol.	Sulfo.	Series.
	degrees	*degrees*	*degrees*	*degrees*	
3-Valeric acid.					
Free acid	+11.4	+19.9	+20.80	+14.32	*l*
Mono-salt	+8.0	+17.3	+13.65	+17.84	
Di-salt			+19.90	+16.06	
4-Valeric acid.					
Free acid	+16.5	+7.1	+6.87	−3.53	*l*
Mono-salt	+3.8	+5.4	+2.43	−3.26	
Di-salt			+2.40	−5.33	

anions of the hydroxy, halogen, thiol, and sulfo acids substituted in positions (3) or (4).

From Table I it is seen that levo-3-thiolvaleric acid, on passing from the undissociated acid to the mono-ion $\left(R\begin{smallmatrix}COO-\\SH\end{smallmatrix}\right)$ shows a change of rotation to the right, and the latter on passing to the di-ion $\left(R\begin{smallmatrix}COO-\\S-\end{smallmatrix}\right)$ shows a change of rotation to the left. The corresponding sulfo acid is levorotatory and on passing to the mono-ion $\left(R\begin{smallmatrix}COOH\\SO_2O-\end{smallmatrix}\right)$ changes its rotation to the left and this on further

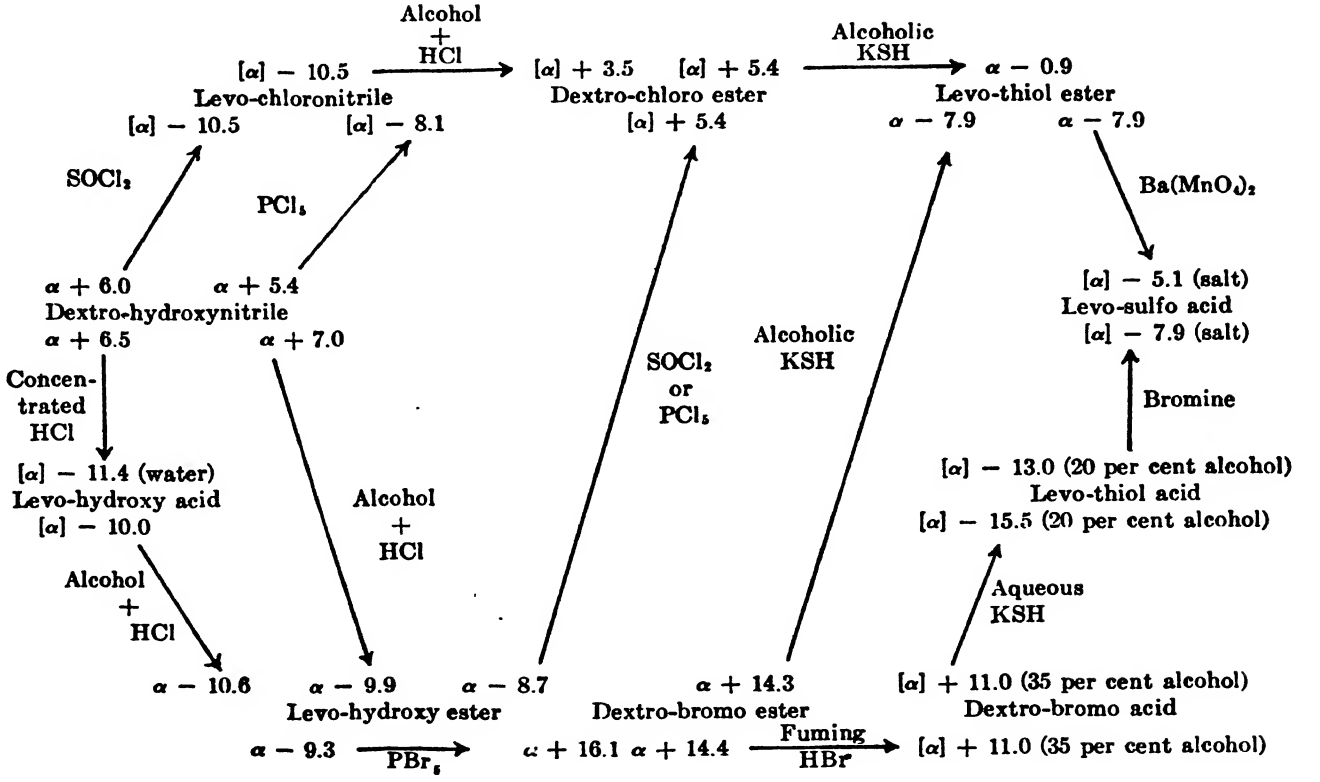
[3] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165, 569.

ionization to the di-ion  changes its rotation to the right. Thus in each case the ionization of the carboxyl leads to a similar change of rotation. This behavior is identical with that of the corresponding acids substituted in position (2).

Levo-4-thiolvaleric acid, on passing to the mono-ion  changes its rotation to the right and the latter on further ionization to the di-ion  does not change perceptibly. The corresponding sulfo acid is dextrorotatory and on conversion into the mono-ion  changes its rotation to the left, and this on further ionization to the di-ion  changes its rotation to the right. Also in this case the behavior is identical with that of the acids substituted in position (2). Thus, the behavior of the 3-thiol- and 3-sulfobutyric acids remains the only exception.

Thus, on the basis of data on the rotations of thiol- and sulfovaleric acids, it seems to us warranted to say that the halogenation of the hydroxy acids was accompanied by a Walden inversion. Also the substitution of the halogen by the thiol group was accompanied by a Walden inversion. As regards the 3-substituted butyric acid, it seems preferable to postpone judgment until further information is obtained concerning the causes of the exceptional behavior of this substance. Work in this direction is in progress.

It may be mentioned here that difficulties were encountered in the early attempts to substitute the hydroxyl of the free acid by halogen. The difficulty was due to the ready lactonization of 3-substituted acids. To overcome the difficulty the nitrile was halogenated prior to its conversion into the free acid. The chloronitrile was readily converted into the chloro ester and this again into the thiol ester, which then was oxidized to the sulfo ester which in the same process was hydrolyzed to the corresponding acid. There were two unsatisfactory features in this set of reactions. First, the reac-



The rotation without solvent are all for 1 dm. tubes. Unless otherwise specified, the solvent used in determining the above rotations was ether. The levo-hydroxy acid used for the preparation of the levo-hydroxy ester was not pure.

tion of chlorination was accompanied by a high degree of racemization. Second, the saponification of the thiol ester did not proceed satisfactorily. All difficulties, however, were overcome when the hydroxy ester was brominated and when the bromo acid was converted into the thiol acid. The entire cycle of reactions is given in the accompanying diagram.

Several points brought out on this diagram are worthy of note. First, the fact that the conversion of the hydroxy- or of the chloronitrile into the corresponding acid or ester is accompanied by a change of direction of rotation. This change of rotation is due to the change in polarity of the radicle affected by the reaction. Second, the reaction of halogenation of the nitrile, ester, or acid leads to a halogenated acid of identical configuration. Third, the halogen acid has the identical configuration regardless of the reagent employed for halogenation.

In the experiments with 4-hydroxyvaleric acid, all reactions proceeded smoothly save one; namely, on treatment of ethyl-4-chlorovalerate with potassium hydrogen sulfide, the resulting product was a mixture of the thiol ester with the thiolactone. The latter rotated in the direction opposite to that of the ester. To verify this conclusion pure 4-thiovalerolactone was prepared from 4-thiolvaleric acid.

EXPERIMENTAL.

Part I. β-Substituted n-Valeric Acids.

Levo-β-Hydroxyvaleric Acid.[4]—Chloromethylethyl ketone prepared by the chlorination of methylethyl ketone was converted into hydroxymethylethyl ketone by the usual method. This compound was reduced to butylene glycol by fermenting bakers' yeast. The best yield of the glycol was 77 gm. from 100 gm. of hydroxymethylethyl ketone. The specific rotations of the glycol were as follows:

$$[\alpha]_D^{20} = \frac{+\ 0.87^\circ \times 100}{1 \times 6.00} = +\ 14.5^\circ, \text{ in absolute alcohol.}$$

$$[\alpha]_D^{20} = \frac{+\ 0.08^\circ \times 100}{2 \times 17.3} = +\ 0.23^\circ, \text{ in water.}$$

[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxiv, 343. We are indebted to Dr. H. L. Haller for the chloromethylethyl ketone used in these experiments.

The glycol ($[\alpha]_D^{20} = +12.4°$ in alcohol) was treated with 1 equivalent of dry hydrogen bromide. From 20 gm. of the glycol 20 gm. of bromohydrin were obtained. The bromohydrin showed the following rotations.

$$\alpha_D^{20} = -10.31°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{-0.72° \times 100}{1 \times 10.0} = -7.2°, \text{ in ether.}$$

The bromohydrin ($\alpha_D^{20} = -5.08°$ without solvent in a 1 dm. tube) was refluxed with potassium cyanide in methyl alcohol. The product, β-hydroxyvaleronitrile, showed the following rotations.

$$\alpha_D^{20} = +8.02°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{+1.00° \times 100}{1 \times 10.0} = +10.0°, \text{ in ether.}$$

The rotation of the free acid was obtained as follows: A sample of the sodium salt of β-hydroxyvaleric acid obtained by saponification of the nitrile ($\alpha_D^{20} = +6.5°$ without solvent in a 1 dm. tube) was treated with 1 equivalent of hydrochloric acid. This solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.48° \times 100}{2 \times 2.11} = -11.4°.$$

Levo-β-Chlorovaleronitrile.—Since the halogenation of the free hydroxy acid as well as of its salt was not satisfactory, the nitrile was chlorinated.

1. By Means of Thionyl Chloride.—To 5 gm. of dextro-β-hydroxyvaleronitrile ($\alpha_D^{20} = +5.98°$ without solvent in a 1 dm. tube) 7.2 gm. (1.2 mols) of thionyl chloride were slowly added under cooling with an ice-salt mixture. The solution was allowed to stand for 1 hour at room temperature and was then heated on the steam bath with a reflux condenser until sulfur dioxide was no longer evolved (about 15 minutes). The reaction mixture was cooled and poured into about the same quantity of crushed ice and shaken to decompose the unchanged thionyl chloride. The mixture was then extracted with ether. The ethereal extract was washed successively with ice water,

with dilute sodium carbonate solution, and with water. After drying with anhydrous sodium sulfate, the ether was removed and the residue was fractionated under reduced pressure (10 mm.). The nitrile boiled at 69–70° and showed the following rotation.

$$[\alpha]_D^{20} = \frac{-1.05° \times 100}{1 \times 10.00} = -10.5°, \text{ in ether.}$$

It is soluble in ether, chloroform, and alcohol, slightly soluble in petrolic ether, and insoluble in water. It analyzed as follows:

0.1194 gm. substance: 0.1528 gm. AgCl.

C_6H_8NCl.	Calculated.	Cl 30.21.
	Found.	" 31.66.

2. By Means of Phosphorus Pentachloride.—10 gm. (1.2 mols) of phosphorus pentachloride were added in small portions to 4 gm. of dextro-β-hydroxyvaleronitrile ($\alpha_D^{20} = +5.35°$ without solvent in a 1 dm. tube) under cooling with an ice-salt mixture. The reaction product was allowed to stand at room temperature until all the pentachloride had disappeared. It was then poured into ice water and the mixture was extracted with ether. The ethereal extract was treated as in the preceding preparation. The nitrile boiled at 68–70° (p = 10 mm.) and gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.55° \times 100}{2 \times 3.4} = -8.1°, \text{ in ether.}$$

The saponification of the chloronitrile and the thiolnitrile (which was prepared by adding alcoholic potassium hydrogen sulfide to the chloro compound) was unsatisfactory, although several methods were tried. Finally the chloronitrile was converted into the ester of the chloro acid as described below.

Levo-Ethyl-β-Hydroxyvalerate.

1. From Levo-β-Hydroxyvaleric Acid.—12 gm. of the levo-hydroxy acid ($[\alpha]_D^{20} = -10°$, in ether), obtained as a syrup by saponification of the hydroxynitrile, were treated with 40 cc. of dry ethyl alcohol containing 1.5 per cent of hydrochloric acid. The solution was gently boiled for 3 hours under a reflux condenser. An aliquot part

of the solution was titrated with sodium hydroxide and the calculated amount of sodium ethylate was added to the remainder under cooling. The filtrate from sodium chloride was concentrated under diminished pressure and the residue was taken up with ether. The ethereal solution was dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at 75.5–77° (p = 9.0 mm.). It gave the following rotations.

$$\alpha_D^{20} = -10.63°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{-1.56° \times 100}{1 \times 10.00} = -15.6°, \text{ in ether.}$$

The ester is soluble in ether, alcohol, petrolic ether, and chloroform and very slightly soluble in water. It analyzed as follows:

4.155 mg. substance: 8.885 mg. CO_2 and 3.575 mg. H_2O.

$C_7H_{14}O_3$. Calculated. C 57.53, H 9.59.
Found. " 58.31, " 9.62.

2. From Dextro-β-Hydroxyvaleronitrile.—15 gm. of dextrohydroxyvaleronitrile ($\alpha_D^{20} = +7.0°$ without solvent in a 1 dm. tube) were dissolved in 75 cc. of absolute (98 to 99 per cent) ethyl alcohol and the solution was saturated with dry hydrogen chloride under cooling with ice. The solution was then heated with a free flame under a return condenser. After 15 minutes ammonium chloride separated. The mixture was boiled 20 minutes longer and then cooled. To the solution some ether was added and the ammonium chloride was filtered off. The filtrate was evaporated at as low a temperature as possible and the residue was taken up in absolute alcohol. The isolation and purification of the ester were carried out as in the preceding preparation. It boiled at 77–79° (p = 10 mm.). The yield of the crude product was 10 gm. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.47° \times 100}{1 \times 10.00} = -14.7°, \text{ in ether.}$$

It analyzed as follows:

6.205 mg. substance: 13.057 mg. CO_2 and 5.335 mg. H_2O.

$C_7H_{14}O_3$. Calculated. C 57.53, H 9.59.
Found. " 57.38, " 9.62.

Dextro-Ethyl-β-Chlorovalerate.

1. From Levo-β-Chlorovaleronitrile.—4 gm. of the levo-chloronitrile ($\alpha_D^{20} = -8.30°$ without solvent in a 1 dm. tube) were dissolved in 32 cc. of absolute alcohol saturated with dry hydrogen chloride and the solution was refluxed for 30 minutes. After 5 minutes ammonium chloride separated. The solution was filtered from ammonium chloride and the alcohol was removed by distillation under reduced pressure. The residue was extracted with chloroform. The chloroform extract was dried over sodium sulfate and evaporated under reduced pressure. During the evaporation crystals appeared in the form of plates having a mother-of-pearl luster. Petrolic ether was added and the solution was filtered. The filtrate from the crystals was concentrated and the residue was fractionated under reduced pressure. The ester boiled at 65–67° (p = 10 mm.). The yield was 2.5 gm. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{+ 0.35° \times 100}{1 \times 10.0} = + 3.5°, \text{ in ether.}$$

It is readily soluble in ether, chloroform, petrolic ether, and alcohol but is insoluble in water. It analyzed as follows:

0.1190 gm. substance: 0.1046 gm. AgCl.
$C_7H_{13}O_2Cl$. Calculated. Cl 21.58.
Found. " 21.74.

The crystals obtained above were purified by dissolving in dry ether and precipitating with petrolic ether. In this state of purity they melted at 100–102° (uncorrected) and gave the following rotation.

$$[\alpha]_D^{20} = \frac{+ 0.36° \times 100}{2 \times 6.00} = + 3.0°, \text{ in chloroform.}$$

The substance analyzed as follows:

0.0988 gm. substance: 0.1028 gm. AgCl.
7.710 mg. " : 0.8377 mg. N (micro Kjeldahl).
$C_5H_{10}OClN$. Calculated. Cl 26.20, N 10.33.
Found. " 25.74, " 10.86.

From the analysis and properties, it seems to us that this substance is β-chlorovaleronamide.

2. From Levo-Ethyl-β-Hydroxyvalerate by Means of Thionyl Chloride.—5 gm. of levo-ethyl-β-hydroxyvalerate ($\alpha_D^{20} = -8.07°$ without solvent in a 1 dm. tube) were treated with 4 gm. (1.2 mols) of thionyl chloride. The isolation and purification were carried out exactly as in the preceding section. It gave the following rotation.

$$\alpha_D^{20} = +6.21°, \text{ without solvent in a 1 dm. tube.}$$

3. From Levo-Ethyl-β-Hydroxyvalerate by Means of Phosphorus Pentachloride.—5 gm. of levo-ethyl-β-hydroxyvalerate ($\alpha_D^{20} = -8.7°$ without solvent in a 1 dm. tube) were dissolved in 10 cc. of dry chloroform. 8.5 gm. (1.2 mols) of phosphorus pentachloride were added under cooling with an ice-salt mixture. The chloro ester was isolated as described above. It boiled at 66.5–67° (p = 10 mm.). It gave the following rotations.

$$\alpha_D^{20} = +6.65°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{+0.54° \times 100}{1 \times 10.0} = +5.4°, \text{ in ether.}$$

The substance analyzed as follows:

0.1197 gm. substance: 0.0991 gm. AgCl.

$C_7H_{13}O_2Cl$. Calculated. Cl 21.58.
Found. " 20.48.

Dextro-Ethyl-β-Bromovalerate.—This compound was prepared because the thiol compound from the chloro ester had a low activity. 10 gm. of levo-ethyl-β-hydroxyvalerate ($\alpha_D^{20} = -9.25°$ without solvent in a 1 dm. tube) were dissolved in 10 cc. of chloroform and 35.5 gm. (1.2 mols) of phosphorus pentabromide were then added in small portions under thorough cooling with an ice-salt mixture. The reaction mixture was allowed to stand at 0° with frequent shaking until all the pentabromide had disappeared. The time necessary was usually 3 hours. The bromo ester was isolated as in the preparation of the chloro body. It boiled at 74–76° (p = 10 mm.). The yield was 10 gm. It gave the following rotations.

$$\alpha_D^{20} = +16.1°, \text{ without solvent in a 1 dm. tube.}$$

$$[\alpha]_D^{20} = \frac{+1.08° \times 100}{1 \times 10.00} = +10.8°, \text{ in ether.}$$

The substance analyzed as follows:

0.1052 gm. substance: 0.0964 gm. AgBr.

$C_7H_{13}O_2Br$. Calculated. Br 38.28.
Found. " 38.99.

It is very soluble in ether, chloroform, petrolic ether, and alcohol but insoluble in water.

Dextro-β-Bromovaleric Acid.—10 gm. of dextro-ethyl-β-bromovalerate ($\alpha_D^{20} = +14.35°$ without solvent in a 1 dm. tube) were treated with 80 cc. of fuming hydrobromic acid under cooling with an ice-salt mixture. The mixture was shaken for 3 days at 10°. It was then poured into about the same quantity of crushed ice and the resulting mixture was extracted several times with chloroform. The chloroform extract was washed with ice water and dried over sodium sulfate. The solvent was evaporated and the residue was fractionated under reduced pressure. After three refractionations the bromo acid boiled at 117–119° (p = 10 mm.). The yield was 3 gm. In another experiment in which the ester was saponified at 40°, the yield was much better, but considerable racemization occurred.

The bromo acid crystallized in glassy plates when allowed to stand overnight at the temperature of solid carbon dioxide. It melted at about 30°. It is soluble in ether, chloroform, alcohol, and petrolic ether, but insoluble in water. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{+ 0.66° \times 100}{4 \times 1.50} = + 11.0°, \quad [M]_D^{20} = + 19.9°, \text{ in 35 per cent alcohol.}$$

To determine the rotation of the sodium salt, 0.2006 gm. of the above bromo acid, which corresponds to 0.225 gm. of sodium salt, was treated with 1 equivalent of sodium hydroxide under cooling and the volume was made up to 15 cc. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{+ 0.51° \times 100}{4 \times 1.50} = + 8.5°, \quad [M]_D^{20} = + 17.3°, \text{ in 35 per cent alcohol.}$$

The bromo acid analyzed as follows:

0.1226 gm. substance: 0.1306 gm. AgBr.

$C_5H_9O_2Br$. Calculated. Br 44.20.
Found. " 45.33.

Levo-Ethyl-β-Thiolvalerate.—7 gm. of dextro-ethyl-β-bromovalerate ($\alpha_D^{20} = +14.25°$ without solvent in a 1 dm. tube) were treated with 33 cc. (3 mols) of alcoholic potassium hydrogen sulfide solution.[5] The mixture was allowed to stand overnight at 0° and then for 2 days at room temperature. It was then heated for 15 minutes on the steam bath to complete the reaction. It was poured into water and the thiol ester was extracted with ether. The ethereal extract was dried over sodium sulfate. After removal of the solvent the residue was fractionated under reduced pressure. It boiled at 75–76°. The yield was 2.5 gm. The residue from the distillation was a thick syrup which was dextrorotatory. The investigation of this substance was not pursued further.

The thiol ester gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.52° \times 100}{1 \times 10.0} = -5.2°, \text{ in ether.}$$

The thiol ester obtained from the dextro-chloro ester ($[\alpha]_D^{20} = +5.4°$ in ether) gave a rotation of only $\alpha_D^{20} = -0.90°$ without solvent in a 1 dm. tube.

2 gm. of the levo-thiol acid ($[\alpha]_D^{20} = -7.75°$ in absolute alcohol) were dissolved in 12 cc. of absolute alcohol and the solution was saturated with dry hydrogen chloride. After the solution had been allowed to stand for 3 days at 0°, the thiol ester was isolated as usual. It boiled at 71–73° (p = 10 mm.) and gave a rotation of

$$[\alpha]_D^{20} = \frac{-0.99° \times 100}{1 \times 10.00} = -9.9° \text{ in ether}$$

but it was not analytically pure.[6]

From the above result it seems to us that in the course of esterification some hydrogen sulfide was split off as in the case of the hydrolysis of the thiol ester which will be described later.

The thiol ester gives a strong nitroprusside reaction but no ferric chloride reaction. It is readily soluble in ether, petrolic ether, chloro-

[5] The alcoholic potassium hydrogen sulfide was prepared by dissolving 20 gm. of potassium hydroxide in 100 cc. of absolute alcohol and saturating the solution with hydrogen sulfide under cooling. The total volume was 113 cc.

[6] $C_7H_{14}O_2S$. Calculated, S 19.75; found, S 16.63.

form, and alcohol, but not in water. The substance analyzed as follows:

0.1155 gm. substance: 0.1619 gm. $BaSO_4$.

$C_7H_{14}O_2S$.	Calculated.	S 19.75.
	Found.	" 19.15.

Levo-β-Thiolvaleric Acid.—The saponification of the thiol ester was not satisfactory either by heating with water or by shaking with concentrated hydrochloric acid in the cold, since it was accompanied by the evolution of hydrogen sulfide. Hence, the thiol acid was prepared from the bromo acid by the usual method.

5.0 gm. of the dextro-bromo acid ($[\alpha]_D^{20} = +7.0°$ in ether) were treated with 10 cc. (10 mols) of 75 per cent aqueous potassium hydrogen sulfide solution. The mixture was allowed to stand for 1 day at 0° and then heated for 15 minutes on the steam bath. It was extracted with ether (when necessary) and acidified with concentrated hydrochloric acid, whereupon the thiol acid separated as an oil. It was extracted with ether and the combined ethereal extract was washed with water. After drying over sodium sulfate, the solvent was evaporated and the residue was fractionated. The thiol acid boiled at 112–113° (p = 10 mm.). It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.24° \times 100}{4 \times 2.00} = -15.5°, \quad [M]_D^{20} = -20.8°, \text{ in 20 per cent alcohol.}$$

For the monosodium salt, 0.2577 gm. of the free acid, which corresponds to 0.3000 gm. of the mono-salt, was treated with 1 equivalent of sodium hydroxide and the volume was made up to 15 cc. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.70° \times 100}{4 \times 2.00} = -8.75°, \quad [M]_D^{20} = -13.65°, \text{ in 20 per cent alcohol.}$$

10 cc. of the above solution were treated with another equivalent of sodium hydroxide and the total volume was made up to 15 cc. This corresponds to 0.2282 gm. of the di-salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.68° \times 100}{4 \times 1.52} = -11.2°, \quad [M]_D^{20} = -19.9°, \text{ in 20 per cent alcohol.}$$

The free acid gave both a ferric chloride reaction (deep indigo blue color) and a nitroprusside reaction. It is easily soluble in ether, petrolic ether, chloroform, alcohol, and very slightly soluble in water. The free acid analyzed as follows:

0.1234 gm. substance: 0.2168 gm. $BaSO_4$.

$C_5H_{10}O_2S$.	Calculated.	S 23.88.
	Found.	" 24.13.

Levo-β-Sulfovaleric Acid.

1. From Levo-Ethyl-β-Thiolvalerate.—3 gm. of the thiol ester ($\alpha_D^{20} = -7.85°$ without solvent in a 1 dm. tube) were dissolved in a mixture of 30 cc. of acetone and 3 cc. of water. To the solution 6.95 gm. of barium permanganate in 500 cc. of acetone were then added and the mixture was warmed on the steam bath near the end of the reaction. The solution was filtered from manganese dioxide and the latter was washed with acetone and with water alternately. The filtrate and the washings were combined and concentrated under reduced pressure. The residue was taken up in a little water and extracted with ether to remove a small quantity of oily substance. The aqueous layer was made up to 50 cc. with alcohol (the resultant alcohol was 40 per cent) and this solution was treated with 7 gm. (2 mols calculated on the starting material) of $Ba(OH)_2 \cdot 8H_2O$. The mixture was gently refluxed for 3 hours. It was then diluted with water and neutralized to litmus with dilute sulfuric acid. It was filtered from barium sulfate and the filtrate was evaporated under reduced pressure. The residue was taken up in hot water and alcohol was then added. The barium salt separated first in amorphous and then in crystalline form. After several recrystallizations it crystallized in the form of white prisms and gave the following rotation. 0.4000 gm. of dry barium salt was dissolved in water and the volume was made up to 5 cc.

$$[\alpha]_D^{20} = \frac{-0.81° \times 100}{2 \times 8.00} = -5.06°, \quad [M]_D^{20} = -16.06°, \text{ in water.}$$

1 equivalent of hydrochloric acid was added and the solution was diluted to 10 cc. This corresponds to 0.314 gm. of monobarium salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.45° \times 100}{2 \times 3.14} = -7.16°, [M]_D^{20} = -17.84°, \text{ in water.}$$

To determine the rotation of the free acid, 0.6768 gm. of dry barium salt was treated with 2 equivalents of hydrochloric acid and diluted to 5 cc. with water. This corresponds to 0.4000 gm. of free acid. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.26° \times 100}{2 \times 8.00} = -7.87°, [M]_D^{20} = -14.32°, \text{ in water.}$$

The barium salt has no melting point. It was quite soluble in water, but insoluble in alcohol. It analyzed as follows:

0.1000 gm. substance: 0.0106 gm. H_2O.
$C_5H_8O_6SBa \cdot 2H_2O$. Calculated. Water of crystallization 10.18.
Found. " " " 10.60.
0.0894 gm. substance: 0.0654 gm. $BaSO_4$ (for Ba).
0.0887 " " : 0.0642 " " (" S).
$C_5H_8O_6SBa$. Calculated. Ba 43.22, S 10.09.
Found. " 43.05, " 9.94.

2. From Levo-β-Thiolvaleric Acid.—1 gm. of the levo-thiol acid was dissolved in 37 cc. (2 equivalents) of 0.4 N barium hydroxide and 9 gm. of barium carbonate (6 equivalents) were then added. The mixture was treated with 4 gm. (a slight excess) of bromine under cooling with ice. The filtrate from the excess of barium carbonate was concentrated to a small volume and alcohol was then added slowly, whereupon the barium sulfonate crystallized out. It was purified by dissolving in a little hot water and precipitating with alcohol. The purified barium salt showed the following rotation.

$$[\alpha]_D^{20} = \frac{-1.20° \times 100}{2 \times 7.58} = -7.92°, \text{ in water.}$$

It analyzed as follows:

0.0943 gm. substance: 0.0688 gm. $BaSO_4$ (for Ba).
0.1426 " " : 0.0930 " " (" S).
$C_5H_8O_6SBa$. Calculated. Ba 43.22, S 10.09.
Found. " 42.93, " 8.96.

Part II. γ-Substituted n-Valeric Acids.

Levo-Ethyl-γ-Chlorovalerate.—Levulinic acid was first prepared and this was reduced to γ-valerolactone according to the directions of Losanitsch.[7] The γ-valerolactone was resolved into its enantiomorphs by means of cinchonidine as described by Levene and Haller.[8] The γ-bromo acid was easily obtained from γ-valerolactone by heating with fuming hydrobromic acid in an autoclave. However, the preparation of the thiol acid from the bromo acid was not successful, although attempted by several methods. The reaction product was always the original substance, *i.e.* γ-valerolactone. Therefore an attempt was made to convert the chloro acid, prepared from its ester as described below, into the thiol acid, inasmuch as the chlorine atom is not so reactive as bromine. Even this reaction was accompanied by the hydrolysis of the chlorine atom. Finally the chloro ester was prepared essentially according to the directions of Noyes[9] for the corresponding racemic form.

35 gm. of levo-γ-valerolactone ($\alpha_D^{20} = -20.32°$ without solvent in a 1 dm. tube) were dissolved in 140 cc. of absolute ethyl alcohol and the solution was saturated with dry hydrogen chloride under cooling with ice. After standing first at 0° and subsequently at room temperature each for 1 day in a stoppered bottle, the solution was poured on crushed ice, whereupon the ester separated as an oil. It was extracted with petrolic ether and the extract was dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at 71–73° (p = 9 mm.) and gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.79° \times 100}{1 \times 4.00} = -19.7°, \text{ in ether.}$$

The chloro ester obtained from the dextro-lactone ($\alpha_D^{20} = +4.5°$

[7] Losanitsch, M. S., *Monatsh. Chem.*, 1914, xxxv, 303.

[8] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165. We are indebted to Dr. H. L. Haller for the active γ-valerolactone used in these experiments.

[9] Noyes, W. A., *J. Am. Chem. Soc.*, 1901, xxiii, 396.

without solvent in a 1 dm. tube) gave a rotation of $\alpha_D^{20} = +4.33°$ without solvent in a 1 dm. tube and analyzed as follows:

0.1046 gm. substance: 0.0920 gm. AgCl.

$C_7H_{13}O_2Cl$.	Calculated.	Cl 21.58.
	Found.	" 21.76.

Levo-γ-Chlorovaleric Acid.—15 gm. of the levo-chloro ester ($\alpha_D^{20} = -5.37°$ without solvent in a 1 dm. tube) were mixed with 150 cc of fuming hydrochloric acid and the mixture was shaken at 10° until solution was complete (2 days). The solution was then allowed to stand for 2 days at 40°. After cooling, the same quantity of crushed ice was added and the solution was extracted with chloroform. The chloroform extract was washed once with ice water and dried over sodium sulfate. After three refractionations the chloro acid boiled at 108–111° (p = 10 mm.). It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.62° \times 100}{2 \times 6.00} = -5.17°, \quad [M]_D^{20} = -7.06°, \text{ in 25 per cent alcohol.}$$

0.2584 gm. of the same sample of the chloro acid was neutralized with 1 equivalent of sodium hydroxide and the volume was made up to 5 cc. under cooling. This corresponds to 0.3000 gm. of sodium salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.41° \times 100}{2 \times 6.00} = -3.42°, \quad [M]_D^{20} = -5.42°, \text{ in 25 per cent alcohol.}$$

The free acid analyzed as follows:

0.1078 gm. substance: 0.1144 gm. AgCl.

$C_5H_9O_2Cl$.	Calculated.	Cl 26.01.
	Found.	" 26.25.

5 gm. of the levo-chloro acid ($\alpha_D^{20} = -6.60°$ without solvent in a 1 dm. tube) were added to 25 cc. of aqueous potassium hydrogen sulfide and the mixture was allowed to stand for 3 days at 0°. An oily substance which separated was extracted with ether and the ethereal extract was dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at

75.5–77° (p = 9 mm.). It contained neither halogen nor sulfur and was found to be γ-valerolactone. It gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.46° \times 100}{1 \times 10.0} = -4.6°, \text{ in ether.}$$

It analyzed as follows:

5.265 mg. substance: 11.650 mg. CO_2 and 3.825 mg. H_2O.

$C_5H_8O_2$. Calculated. C 60.00, H 8.00.
Found. " 60.34, " 8.12.

Dextro-Ethyl-γ-Thiolvalerate.—30 gm. of the levo-chloro ester ($\alpha_D^{20} = -4.5°$ without solvent in a 1 dm. tube) were treated with 145 cc. (2½ mols) of alcoholic potassium hydrogen sulfide solution. After standing at 0° and at room temperature each for 1 day, the mixture was heated at 150° for 2½ hours in a pressure bottle. The solution was cooled and poured into ice water. The ester was extracted with ether. On removal of the ether, a light yellow mobile oil remained which was fractionated under a pressure of 10 mm. The rotations given below are all for 1 dm. tubes.

F I 73–75°	Weight = 5 gm.	$\alpha_D^{20} = -4.95°$
F II 74.5–76.5°	" = 13 "	$\alpha_D^{20} = -3.96°$
F III 76–80°	" = 5 "	$\alpha_D^{20} = 0°$

F III was redistilled.

F I^I 78–79°	$\alpha_D^{20} = -1.45°$
F II^I 79–80°	
F III^I 80–83°	$\alpha_D^{20} = +2.10°$

F III^I was again redistilled.

F I^{II} 77–80°		
F II^{II} 81–82°	Weight = 1 gm.	$[\alpha]_D^{20} = \frac{+0.17° \times 100}{1 \times 10.0} = +1.7°$, in ether.

As above, we obtained dextro- and levorotatory substances which were both neutral to litmus. From the results of analyses and the behavior toward iodine solution the levorotatory substance is the thiolactone and the dextrorotatory is the thiol ester, although the thiolactone was not isolated in a pure state from the above prepara-

tion. To make this conclusion certain, the pure thiolactone was prepared from the thiol acid as described later.

The substances analyzed as follows:

F I	0.1146 gm. substance:	0.1913 gm. $BaSO_4$.	Found.	S 23.28.
F I[II]	0.1174 " "	: 0.2024 " "	"	" 23.68.
F II[II]	0.1037 " "	: 0.1554 " "	"	" 20.59.
	C_4H_8OS (lactone).	Calculated.	S 27.59.	
	$C_7H_{14}O_2S$ (ester).	"	" 19.75.	

The thiol ester has a quite unpleasant odor and gives a strong nitroprusside reaction but no ferric chloride reaction. It is easily oxidized by iodine; *i.e.*, it decolorizes iodine solution. It is readily soluble in ether, petrolic ether, chloroform, and alcohol, but not in water.

Dextro-γ-Thiolvaleric Acid.—20 gm. of the mixture of the dextro-thiol ester and the levo-thiolactone ($\alpha_D^{20} = -19.82°$ without solvent in a 1 dm. tube) were dissolved in 200 cc. of 90 per cent alcohol containing 20 gm. of potassium hydroxide and the solution was heated on the steam bath for 2 hours under a reflux condenser. The excess of alcohol was removed by distillation under reduced pressure. The residue was diluted with ice water and acidified with concentrated hydrochloric acid under cooling, whereupon the thiol acid separated as an oil. It was then extracted with ether and the etheral extract was dried over sodium sulfate. The thiol acid boiled at 121–122°. These operations should be performed as quickly as possible after acidifying. It gave the following rotations.

$$[\alpha]_D^{20} = \frac{+ 0.50° \times 100}{2 \times 4.00} = + 6.25°, \text{ in ether.}$$

$$[\alpha]_D^{20} = \frac{+ 0.42° \times 100}{2 \times 4.09} = + 5.14°, [M]_D^{20} = + 6.87°, \text{ in 20 per cent alcohol.}$$

For the rotation of the mono-salt, 0.5000 gm. of the same sample was treated with 1 equivalent of sodium hydroxide solution and the volume was made up to 5 cc. This corresponds to 0.582 gm. of the acid salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{+ 0.37° \times 100}{2 \times 11.6} = + 1.56°, [M]_D^{20} = + 2.43°, \text{ in 20 per cent alcohol.}$$

To the above solution another equivalent of sodium hydroxide was added and the volume was made up to 10 cc. This corresponds to 0.664 gm. of disodium salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{+ 0.18^\circ \times 100}{2 \times 6.64} = + 1.36^\circ, \; [M]_D^{20} = + 2.40^\circ, \text{ in 20 per cent alcohol.}$$

The thiol acid is very soluble in ether and alcohol, but very slightly soluble in water. It analyzed as follows:

0.1052 gm. substance: 0.1828 gm. $BaSO_4$.

$C_5H_{10}O_2S$. Calculated. S 23.88.
Found. " 23.74.

Levo-γ-Thiovalerolactone.[10]—3 gm. of dextro-γ-thiolvaleric acid ($\alpha^{20} = +4.87^\circ$ without solvent in a 1 dm. tube) were added to 30 cc. of 10 per cent sulfuric acid and the mixture was shaken for 2 days at 40°. It was then extracted with ether and the ethereal extract was washed with water and dried first with sodium sulfate and subsequently with anhydrous potassium carbonate. After removal of the ether, the thiolactone was fractionated under reduced pressure. It boiled at 69–70° (p = 10 mm.). It gave the following rotation.

$$[\alpha]_D^{20} = \frac{- 7.83^\circ \times 100}{1 \times 10.00} = - 78.3^\circ, \text{ in ether.}$$

The thiovalerolactone has a not unpleasant odor. It is neutral to litmus, does not decolorize iodine solution, gives no ferric chloride reaction, but gives a nitroprusside reaction. It is soluble in ether, petrolic ether, glacial acetic acid, chloroform, and alcohol, but insoluble in water. The substance analyzed as follows:

0.1015 gm. substance: 0.2144 gm. $BaSO_4$.

C_5H_8OS. Calculated. S 27.60.
Found. " 29.02.

Levo-γ-Sulfovaleric Acid.—3 gm. of the dextro-γ-thiol acid ($[\alpha]_D^{20} = +6.25^\circ$, in ether) were dissolved in 75 cc. (1 equivalent) of 0.3 N barium hydroxide and 26 gm. of barium carbonate (6 equivalents)

[10] The inactive substance was prepared by Fries from valerolactone and phosphorus pentasulfide. Fries, K., and Mengel, H., *Ber. chem. Ges.*, 1912, xlv, 3410.

were added. The mixture was treated with 10 gm. (6 equivalents) of bromine in small portions under cooling. The filtrate from the excess of barium carbonate was concentrated to a small volume under reduced pressure. To the solution alcohol was added, whereupon the barium sulfonate was precipitated as an amorphous substance. The precipitate was purified by dissolving in a little hot water and precipitating with alcohol. This treatment was repeated four times. The barium salt gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.67^\circ \times 100}{2 \times 20.0} = -1.68^\circ, \quad [M]_D^{20} = -5.33^\circ.$$

3.0573 gm. of the same sample of barium salt were dissolved in 1 equivalent of hydrochloric acid and the volume was made up to 15 cc. This corresponds to 2.400 gm. of mono-salt. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-0.84^\circ \times 100}{4 \times 16.0} = -1.31^\circ, \quad [M]_D^{20} = -3.26^\circ.$$

For the free sulfo acid, 4.1812 gm. of the same substance were treated with 2 equivalents of hydrochloric acid. This corresponds to 2.400 gm. of free acid. The solution gave the following rotation.

$$[\alpha]_D^{20} = \frac{-1.24^\circ \times 100}{4 \times 16.0} = -1.94^\circ, \quad [M]_D^{20} = -3.53^\circ.$$

The barium salt is very soluble in water but not in alcohol. It has no melting point. It analyzed as follows:

0.0908 gm. substance: 0.0652 gm. $BaSO_4$ (for Ba).
0.0919 " " : 0.0673 " " (" S).
$C_4H_8O_4SBa$. Calculated. Ba 43.22, S 10.09.
Found. " 42.25, " 10.06.

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STUDIES ON RACEMIZATION.

VII. THE ACTION OF ALKALI ON CASEIN.

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In previous publications[1] from this laboratory it has been pointed out that the progress of racemization of a given protein at different hydrogen ion concentrations may lead to definite conclusions regarding certain details of the structure of the protein molecule. This expectation is based upon observations on the action of alkali upon peptides and upon ketopiperazines. Dipeptides are not appreciably racemized under the influence of alkali. Higher polypeptides undergo racemization only at a very slow rate. Ketopiperazines, on the other hand, are racemized very rapidly under the proper conditions, but when the concentration of alkali is such that the rate of hydrolysis of the ketopiperazines is very high, the extent of racemization is very low. Hence, if a protein molecule contained ketopiperazines, it would be expected to behave in the following way. On treatment with dilute alkali, it would undergo marked racemization; on treatment with stronger alkali the degree of racemization would be minimal. A result of this type was actually observed in the case of gelatin. Table I gives a summary of the data which were reported in a previous article.[2]

The results of the observations on the action of alkali on casein are not so easily interpreted as those in the case of gelatin. The progress of racemization under the influence of alkali in higher dilution (0.5 N

[1] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661; *J. Gen. Physiol.*, 1925, viii, 183; *J. Biol. Chem.*, 1926, lxviii, 277; 1926, lxx, 219. Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, 1927, lxxii, 815. Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, 1927, lxxiv, 715. Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 1928, lxxvi, 299.

[2] Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, 1927, lxxiv, 715.

and 1.0 N) is such as would be expected from a structure containing ketopiperazines. On treatment with more concentrated alkali (5.0 N), casein, in contrast to gelatin and to ketopiperazines, shows a still higher rate of racemization than with dilute alkali.

From Table IV and Fig. 1 it is seen that with 1.0 N sodium hydroxide casein in 24 hours undergoes 41 per cent racemization calculated on the basis of total possible racemization or 47 per cent on the basis of maximum observed racemization. This observation means that of all the amino acids which are linked in a manner permitting racemiza-

TABLE I.

Racemization of Gelatin by Sodium Hydroxide.

NaOH	Time.	Rotation.	Racemization.
	days	*degrees*	
0.1 N	Control.	−0.38	−
	1	−0.69	+
	2	−0.79	+
1.0 N	Control.*	−0.36	−
	1	−1.13	+
	2*	−0.96	+
3.0 N	Control.	−0.23	−
	1	−0.20	−
	2	−0.18	−
	4	−0.13	−

* Through a typographical error these experiments were indicated in the table in the original paper as 0.1 N NaOH. Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, 1927, lxxiv, 724.

tion, 47 per cent are racemized in 24 hours. Ketopiperazines which have thus far been studied undergo as much as 80 per cent racemization in the course of 24 hours. With 5.0 N sodium hydroxide casein in 24 hours undergoes 66 per cent racemization calculated on the basis of maximum observed racemization. This degree of racemization is of the order of magnitude of the racemization of ketopiperazines, but for the ketopiperazines thus far studied, the rate of hydrolysis under the influence of strong alkali is so high that racemization does not set in at all. *Hence it seems that casein is not composed of keto-*

piperazines of the type thus far studied, nor is it a simple polypeptide of the type thus far studied. Two alternative assumptions may be offered to explain the conduct of casein: first, that the ketopiperazines in casein are more stable than the ketopiperazines thus far studied; or, second, that the order of linkage of the amino acids is such as to facilitate racemization.

It is certain that in casein, as in ketopiperazines or in peptides, racemization precedes cleavage. This conclusion is warranted by the fact that the unhydrolyzed part of casein shows the same degree of

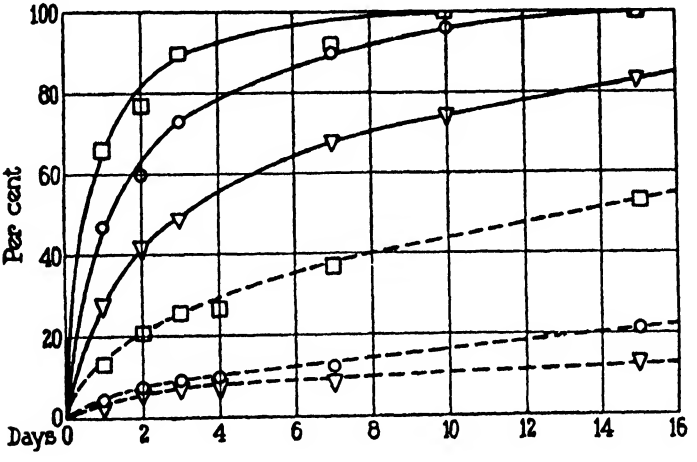

FIG. 1. The racemization and hydrolysis of casein by sodium hydroxide at 25°. The racemization curves are drawn in solid lines, the hydrolysis curves in broken lines. △ = 0.5 N NaOH; ○ = 1.0 N NaOH; □ = 5.0 N NaOH.

racemization as the partially hydrolyzed part (caseoses), as seen from Tables IX and X.

The greater stability of casein as compared with gelatin is clearly seen from a comparison of Tables V and VI. Furthermore, as is seen from Table VII, it was not possible to increase the velocity of alkaline hydrolysis without increasing the rate of racemization. It was hoped that by raising the temperature of the reaction to 125° the rate of hydrolysis might be increased to such an extent that racemization would be avoided. However, under these conditions complete racemization was observed after 2 hours, whereas hydrolysis apparently was not yet complete in the same interval of time. In

this connection it may be recalled that Levene and Simms,[8] in their work on the relation of structure to the rate of hydrolysis of peptides, have found that peptides methylated on the nitrogen atom possess much greater stability than ordinary peptides.

Further work on synthetic peptides and on synthetic ketopiperazines, as well as work on a series of proteins of distinctly different composition, is required in order that the observations on the action of alkali on casein and on other proteins may be fully interpreted.

EXPERIMENTAL.

General Procedure.—The experimental procedure employed in the present investigation is essentially the same as that described in the previous paper. However, the hydrolyses were effected with hydrochloric acid instead of sulfuric acid because of the greater efficiency of the former. Likewise, these hydrolyses were carried out at a higher temperature in order to reduce the time necessary to reach a constant, maximum amino nitrogen ratio.

The protein employed was a uniform sample of Kahlbaum casein (Hammarsten). It gave the following analysis (calculated as dry material).

C 53.56, H 6.91, P 0.63, S 1.13, N 14.94, NH_2 0.80.
Ash 2.16. Moisture 8.41.

A uniform sample of norit was used as decolorizing agent.

The acid hydrolyses were carried out at 125° in a large glycerol thermostat equipped with a steam preheater and heated by an intermittent electric heating unit controlled by a thermoregulator. Thorough stirring was maintained by means of a motor. The temperature was constant within ±0.5°.

The hydrolyses were carried out with 1.000 gm. (calculated as dry substance) of casein, 0.400 gm. of norit, and 10.0 cc. of standard acid, the mixtures being sealed in Pyrex test-tubes. After hydrolysis at 125° the solutions were filtered and diluted to 25.0 cc. The rotations of these solutions were measured in 4.00 dm. open tubes at 25° for the wave-length 5892 Å. All rotations were corrected to a con-

[8] Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, 1924, lxi, 445; 1926, lxx, 253. Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1924–25, lxii, 711.

centration of 0.956 mg. of nitrogen per cc. in the solutions used for determining the amino nitrogen ratios, which were prepared by diluting 4.00 cc. of the rotation solutions to 25.0 cc.

Acid Hydrolysis at 125°.—A series of experiments with different concentrations of acid and different periods of heating was run in order to determine the optimum conditions for complete hydrolysis of the protein. In our choice of hydrochloric acid and a temperature of 125°, we were guided by the data on gelatin which we have reported previously.

Samples of casein equivalent to 1.000 gm. of dry substance with 0.400 gm. of norit and 10.0 cc. of standard acid were sealed in Pyrex test-tubes. The tubes were shaken to insure thorough mixing and were then heated for the required periods of time in the 125° thermostat.

The contents of the tubes were filtered through small folded filters into 25.0 cc. flasks and the filters were washed carefully with distilled water. In the experiments with 5.0 N acid, the flasks were then filled to the mark with distilled water, the resulting solutions thus being 2.0 N with respect to the acid. In the experiments with 1.0 N and with 3.0 N acid the necessary quantity of strong acid was added to bring the final 25.0 cc. solutions to a concentration of 2.0 N acid, in order that the rotations might be strictly comparable. The rotations of these solutions were then read.

For analysis, 4.00 cc. of the rotation solutions were neutralized to phenolphthalein with alkali and diluted to 25.0 cc. Total nitrogen was determined on 10.0 cc. samples (Kjeldahl) and amino nitrogen on 2.00 cc. samples (micro Van Slyke, 15 minutes).

The data are given in Table II. Each value is the mean of at least two independent experiments. The agreement between independent experiments was very good, the error in the rotations being $\pm 0.03°$ and in the amino nitrogen ratios ± 0.7 per cent in the solutions in which hydrolysis was complete or nearly complete. In the experiments with a lower degree of hydrolysis, the experimental error was greater. The degrees of hydrolysis recorded in Column 4 are calculated on the assumption that complete hydrolysis is represented by an amino nitrogen ratio of 76.2 per cent and that the amino nitrogen ratio of the original material is 5.4 per cent.

A series of experiments with quantities of norit varying from 0 to 0.800 gm. showed that the use of this substance as a decolorizing agent introduced no appreciable error in the amino nitrogen ratios or in the rotations. (The rotations in the experiments with less than 0.200 gm. of norit could not be read because of their strong red color.)

An inspection of the data shows that hydrolysis is complete on heating with 5.0 N acid for 4 hours. These conditions were therefore adopted as standard conditions for the racemization experiments.

TABLE II.

Hydrolysis of Casein by Hydrochloric Acid at 125°.

Acid. (1)	Time. (2)	$\frac{\text{Amino N}}{\text{Total N}}$. (3)	Hydrolysis. (4)	α_D^{25} corrected. (5)
	hrs.	*per cent*	*per cent*	*degrees*
1.0 N	2	46.5	58.1	−4.15
	6	56.2	71.8	−1.15
	24	62.0	80.0	+0.50
3.0 N	2	65.5	84.9	+0.88
	6	72.0	94.1	+1.61
	24	76.4	100.3	+1.78
5.0 N	2	70.3	91.7	+1.60
	4	75.8	99.5	+1.86
	6	76.3	100.1	+1.80
	24	76.1	99.9	+1.75

The data for the calculation of the rotation of completely hydrolyzed casein in excess of acid are given in Table III.

Racemization by Alkali at 25°.—The racemization experiments were carried out exactly as in the case of gelatin, except that 0.5 N, 1.0 N, and 5.0 N sodium hydroxide were used instead of 0.1 N, 1.0 N, and 3.0 N. Samples of casein equivalent to 1.000 gm. of dry substance were dissolved in 25.0 cc. of standard alkali, 1 drop of octyl alcohol being used to prevent foaming during solution. The solutions were then allowed to stand at 25° for the times indicated.

Each solution was then neutralized to litmus with hydrochloric acid. Near the neutral point a curdy, sticky solid, the racemized

TABLE III.

Calculation of Rotation of Mixture of Amino Acids Obtained by Hydrolysis of Casein.

Amino acid.	Per cent in casein. (1)	$[\alpha]_D^{25}$ in excess of acid. (2)	Rotation in hydrolyzed casein. (3)	Racemization, racemized casein. (4)	Racemization, racemized caseose. (5)
Glycine	0.45				
Alanine	1.85	+10.4	+0.03	Complete (?).	Complete (?).
Valine	7.93	+28 8	+0.37	Partial.	Partial.
Leucine	7.92	+15.8	+0.20	"	"
Isoleucine	1.43	+40.6	+0.09		
Phenylalanine	3.88	−7.1	−0.04	Complete.	Complete.
Tyrosine	5.70	−8.5	−0.08	"	"
Serine	0.43	+14.4	+0.01		
Cystine	0.02	−224.0	−0.01		
Aspartic acid	4.10	+25.7	+0.17	Complete.	Complete.
Glutamic "	21.77	+34.9	+1.21	"	"
Hydroxyglutamic acid	10.50	+16.3	+0.27		
Lysine	7.72	+15.5	+0.19	Complete.	Complete.
Arginine	4.84	+21.2	+0.16	"	"
Histidine	3.39	+9.5	+0.05	"	"
Tryptophane	1.70	−13.5	−0.04		
Proline	8.70	−48.6	−0.68	None.	None.
Hydroxyproline	0.23	−49.2	−0.02		
Calculated rotation of completely hydrolyzed casein			+1.88		

Column 1 shows the percentages of the amino acids contained in casein as given by Gortner.[4] Column 2 shows the specific rotations of the naturally occurring amino acids, compiled from the literature. Some of these values have been changed from those recorded in the paper on gelatin.[2] Column 3 gives the calculated rotation of a mixture of amino acids corresponding to the composition of casein. This rotation is calculated for 1.000 gm. of casein in excess of acid measured in a 4 dm. tube; *i.e.*, the actual experimental conditions. Columns 4 and 5 give the results of Dakin and Dudley's[5] investigation of the racemization of casein.

[4] Gortner, R. A., in Sutermeister, E., Casein and its industrial applications, New York, 1927, 19.

[5] Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 263.

casein described by Dakin and Dudley,[5] separated. The solution, without removal of the solid, was evaporated to dryness in a Pyrex test-tube under reduced pressure at 50°. It was necessary to add a few drops of octyl alcohol from time to time to prevent foaming. Each residue was then treated with a small quantity of 98 per cent alcohol and again evaporated to dryness. To each residue were added 0.400 gm. of norit and 10.0 cc. of 5.0 N hydrochloric acid and the tubes were sealed. The hydrolysis for 4 hours at 125° and the subsequent operations were then carried out exactly as described under acid hydrolysis.

Control experiments for the racemizations were run by hydrolyzing for 4 hours at 125° 1.000 gm. samples of casein with 0.400 gm. of norit and 10.0 cc. of 5.0 N hydrochloric acid plus the quantities of sodium chloride equivalent to 25.0 cc. of 0.5 N, 1.0 N, and 5.0 N sodium hydroxide, respectively; *viz.*, 0.73 gm., 1.46 gm., and 7.30 gm. In the racemization experiments with 5.0 N sodium hydroxide and in the corresponding controls there was a residue of solid sodium chloride in the hydrolysis tubes.

The data are given in Table IV. The percentage racemizations calculated on the maximum observed racemization (final column) are plotted in Fig. 1.

Alkaline Hydrolysis at 25°.—The rate of hydrolysis of casein under the conditions employed in the racemizations was followed in a separate series of experiments. Samples of protein equivalent to 2.000 gm. of dry substance were dissolved in 50.0 cc. of standard alkali, 1 drop of octyl alcohol being added to each solution to prevent foaming during solution. The solutions were allowed to stand at 25°. The progress of hydrolysis was followed by the analysis of 4.00 cc. samples taken at the time intervals shown. These samples were neutralized to phenolphthalein and diluted to 25.0 cc. Total nitrogen and amino nitrogen (30 minutes) were determined as in the acid hydrolyses.

The results are given in Table V. In calculating the degree of hydrolysis from the amino nitrogen ratios, 76.2 per cent was taken as the amino nitrogen ratio of completely hydrolyzed casein and 5.4 per cent as the amino nitrogen ratio of the original material. The percentage hydrolyses are plotted in Fig. 1.

Alkaline Hydrolysis of Gelatin at 25°.—For comparison, the hydrolysis of gelatin by sodium hydroxide at 25° was studied in a series of experiments similar to those used for casein.

The results are given in Table VI. In calculating the degree of hydrolysis from the amino nitrogen ratios, 70.0 per cent was taken

TABLE IV.

Racemization of Casein by Sodium Hydroxide at 25°.

Alkali.	Time.	Total N per cc.	Amino N / Total N	α_D^{25} corrected.	Racemization calculated on complete racemization.	Racemization calculated on maximum observed racemization.
	days	*mg.*	*per cent*	*degrees*	*per cent*	*per cent*
0.5 N	Control.	0.938	75.7	+1.82		
	1	0.903	75.1	+1.38	24	28
	2	0.882	75.3	+1.16	36	42
	3	0.861	75.0	+1.05	42	49
	7	0.759	74.3	+0.74	59	68
	10	0.762	75.6	+0.65	64	74
	15	0.757	75.4	+0.51	72	83
1.0 N	Control.	0.907	76.2	+1.85		
	1	0.878	75.9	+1.10	41	47
	2	0.665	74.0	+0.88	52	60
	3	0.829	74.5	+0.67	64	73
	7	0.860	74.3	+0.40	78	90
	10	0.762	75.0	+0.30	84	96
	15	0.801	74.5	+0.23	88	100
5.0 N	Control.	0.917	75.2	+1.80		
	1	0.826	75.1	+0.77	57	66
	2	0.841	73.8	+0.60	67	77
	3	0.868	73.9	+0.40	78	90
	7	0.718	75.7	+0.37	79	92
	10	0.889	73.3	+0.24	87	100
	15	0.834	74.6	+0.25	86	100

as the amino nitrogen ratio of completely hydrolyzed gelatin and 3.3 per cent as the amino nitrogen ratio of the original material.

Racemization of Casein by Alkali at 125°.—Samples of casein equivalent to 1.000 gm. of dry substance were dissolved in 25.0 cc. of 1.0 N sodium hydroxide and the solutions sealed in Pyrex bomb

tubes. These tubes were heated for 2 hours at 125°. The amino nitrogen ratios were determined on 4.00 cc. samples. The remain-

TABLE V.

Hydrolysis of Casein by Sodium Hydroxide at 25°.

Time.	Degree of hydrolysis.		
	0.5 N NaOH.	1.0 N NaOH.	5.0 N NaOH.
days	*per cent*	*per cent*	*per cent*
1	3.4	4.0	13.1
2	6.5	6.9	20.8
3	7.5	8.5	25.8
4	7.8	9.6	26.6
7	8.1	13.1	37.2
15	13.1	21.4	52.9
30	18.4	29.2	67.4

TABLE VI.

Hydrolysis of Gelatin by Sodium Hydroxide at 25°.

Time.	Degree of hydrolysis.		
	0.5 N NaOH.	1.0 N NaOH.	5.0 N NaOH.
days	*per cent*	*per cent*	*per cent*
1	11.2	28.6	77.4
2	20.2	41.9	83.1
4	32.1	54.9	88.6
7	38.8	63.6	92.7
10	50.0	71.4	98.7
15	52.4	77.7	100.8

TABLE VII.

Racemization of Casein by Sodium Hydroxide at 125°.

Experiment No.	Amino N ratio before acid hydrolysis.	Total N per cc. after acid hydrolysis.	Amino N ratio after acid hydrolysis.	α_D^{25} corrected.
	per cent	*mg.*	*per cent*	*degrees*
1	68.4	0.668	70.1	+0.01
2	67.2	0.427	72.7	0
3	66.9	0.540	70.7	−0.03

ing solutions were treated exactly as described in the racemization experiments. The final acid solutions, after hydrolysis, were op-

TABLE VIII.

Analysis of Racemized Casein.

	Weight.	C	H	P	S	N	NH_2	Moisture.	Ash.
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Non-racemized casein		53.56	6.91	0.63	1.13	14.94	0.80	8.41	2.16
Racemized casein.									
Experiment I	2.74	52.03	6.58	0	0.26	12.83	0.77	8.60	2.73
Experiment II	3.04	52.99	6.79			13.05	0.87	5.64	3.30

TABLE IX.

Hydrolysis of Racemized Casein by Hydrochloric Acid at 125°.

	$\frac{\text{Amino N}}{\text{Total N}}$.	α_D^{25} corrected.
	per cent	*degrees*
Non-racemized casein. (See Table II.)	75.8	+1.86
Corresponding racemization experiment. (See Table IV.)	74.5	+0.67
Racemized casein.		
Experiment I	77.2	+0.67
Experiment II	76.4	+0.67

TABLE X.

Soluble Fraction from Racemized Casein.

	Before hydrolysis.		After hydrolysis.		
	Total N per cc.	$\frac{\text{Amino N}}{\text{Total N}}$.	Total N per cc.	$\frac{\text{Amino N}}{\text{Total N}}$.	α_D^{25} corrected.
	mg.	*per cent*	*mg.*	*per cent*	*degrees*
Non-racemized casein. (See Table II.)		5.4		75.8	+1.86
Corresponding racemization experiment. (See Table IV.)		8.5		74.5	+0.67
Racemized casein.					
Experiment I	2.058	20.0	0.794	74.5	+0.61
Experiment II	1.848	24.2	0.717	73.0	+0.57

tically inactive within the experimental error. The data are recorded in Table VII.

Insoluble and Soluble Fractions from Racemized Casein.[5]—Samples of casein equivalent to 5.000 gm. of dry substance were dissolved in 125.0 cc. of 1.0 N sodium hydroxide. After having stood 3 days at 25°, the solutions were made acid to litmus with glacial acetic acid. The gummy precipitates of racemized casein settled to the bottom and the supernatant liquids, containing racemized caseose and other soluble materials, were decanted.

The precipitates were washed by decantation with distilled water and dried in a vacuum desiccator. The analyses are recorded in Table VIII.

Samples of 1.000 gm. were hydrolyzed at 125° for 4 hours with 10.0 cc. of 5.0 N hydrochloric acid and 0.400 gm. of norit. The data are recorded in Table IX.

The supernatant liquids containing racemized caseose and other soluble materials were concentrated to dryness under diminished pressure at 50° and the residues were dried in a vacuum desiccator. Each residue was then dissolved in 20 cc. of 5.0 N hydrochloric acid, filtered, and diluted to 25.0 cc. with 5.0 N hydrochloric acid. Samples of 4.00 cc. of each solution were analyzed for total nitrogen and amino nitrogen. The analyses are recorded in Table X.

Samples of 10.0 cc. of each solution were sealed in Pyrex test-tubes with 0.400 gm. samples of norit and hydrolyzed at 125° for 4 hours. The solutions were filtered and diluted to 25.0 cc. and the rotations were read. Samples of 4.00 cc. were then diluted to 25.0 cc. and analyzed. The data are recorded in Table X.

SUMMARY.

1. The rotations and amino nitrogen ratios have been determined for the mixture of amino acids obtained from casein by hydrolysis with hydrochloric acid at 125°. Complete hydrolysis, together with maximum rotation, is attained when the protein is heated 4 hours with 5.0 N acid.

2. The rotations and amino nitrogen ratios have been determined for the mixture of amino acids obtained by acid hydrolysis of casein which had previously been subjected to the action of 0.5 N, 1.0 N,

or 5.0 N sodium hydroxide at 25° for different periods of time. Racemization increases with the strength of the alkali and with the time.

3. The rates of hydrolysis at 25° of casein and gelatin by 0.5 N, 1.0 N, and 5.0 N sodium hydroxide have been determined.

4. From the results of (2) and (3) it appears that casein is not composed of ketopiperazines of the type thus far studied, nor is it a simple polypeptide of the type thus far studied. The behavior of casein may be explained by assuming that the ketopiperazines contained in it are more stable than those previously studied or that the order of linkage of the amino acids is such as to facilitate racemization.

5. When casein is heated with 1.0 N sodium hydroxide at 125°, complete racemization occurs. Hence, under these conditions it is impossible to increase the rate of hydrolysis of the ketopiperazines sufficiently to avoid racemization.

6. An investigation of the soluble and insoluble fractions of racemized casein has shown that racemization precedes hydrolysis.

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PREPARATION OF ELECTROLYTE-FREE GELATIN.

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The method described here is a modification of the one employed by Loeb. His method consisted principally in bringing powdered gelatin, by means of acetic acid, to a pH slightly below the isoelectric point and then washing it several times with distilled water. This process brought about a complete removal of various cations. No special care was taken to remove anions except so far as they were replaced by the acetate ion of the acetic acid. In our modified method, of which a brief description was given in a former publication,[1] the gelatin is treated, in addition to acetic acid, also with dilute alkali, and then washed at pH 4.7 with H_2O, thus bringing about a removal of both cations and anions. The details of the process are as follows.

1000 gm. of Cooper's non-bleached powdered gelatin, the fine dust of which has been removed by screening it through an 80 mesh sieve, are soaked for 1 hour, with constant stirring, in 20 liters of M/128 acetic acid, at a temperature of 5° to 10°C. A glazed 5 gallon earthenware jar immersed in a water bath cooled by means of a coil from a refrigerating machine is employed as a container. The gelatin is washed three times with cold distilled water by allowing the gelatin grains to settle, removing the sypernatant liquid as thoroughly as possible, and then refilling the container. The supernatant liquid is conveniently removed by the use of an inverted Büchner funnel, 14 cm. in diameter, connected by means of a long rubber tubing to a 4 liter suction flask and then to a suction pump. A padding made of towel cloth is spread on the funnel in order to filter out the gelatin. The suspension of gelatin is stirred for about 20 minutes in the washing liquid before renewing it.

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 354.

After the last washing the container is refilled to the mark with cold H_2O and a definite amount of strong NaOH solution of a known strength (80 cc., 5 M) is added to the gelatin so as to bring the total strength to about M/50 alkali. The NaOH solution is added slowly while the gelatin is thoroughly stirred. The gelatin is allowed to remain in the alkaline solution for 1 hour. The supernatant solution is then removed and the gelatin washed three times with cold H_2O in the same manner as after the treatment with acetic acid. But care is taken to keep track of the amount of NaOH removed by the washings by measuring the volume of liquid decanted and also titrating samples of it with HCl. Thus the total amount of NaOH left in the gelatin solution is definitely found.

Acetic acid of a known strength, in amount double that of the *NaOH left* in the gelatin, is then added to the gelatin. This brings the gelatin to pH 4.7. The gelatin mass, which had been greatly swollen in the alkali, shrinks considerably at this stage, and the removal of electrolytes by washing with cold distilled water proceeds rapidly. After four or five washings the specific conductivity of the settled gelatin when melted is from 3 to 5×10^{-5} reciprocal ohms at 35°C. Its concentration is about 17 gm. per 100 cc. solution. The gelatin, after it has been sufficiently washed and the washings removed, is stirred up with about 5 liters of 95 per cent alcohol for about 15 minutes. It is afterwards transferred to a large Büchner funnel, perfused once or twice with fresh alcohol and then with ether. It dries easily in the air when spread out in a thin layer on filter paper. A solution of 2.3 gm. of the dry gelatin in distilled water had a pH of 4.84 and a specific conductivity of 1.5×10^{-5}. The specific conductivity of the water used was 3.4×10^{-6}.

Further washings do not affect the conductivity to any considerable degree. One of the effects of too much washing is the removal of the final traces of diffusible anions which are required to keep the water in which the gelatin is dissolved at pH 4.7, with the result that the gelatin in dilute solutions is negatively charged.[2] If it is desirable to

[2] Dhéré (Dhéré, C., *Kolloid Z.*, 1927, xli, 315) found this to be the fact also in case of purification of gelatin by electrodialysis. He concludes that the isoelectric gelatin of Loeb is not isoelectric at all but a combination of gelatin with ions. Apparently Dhéré fails to appreciate the fact that since at the isoelectric point of

keep the pH at the isoelectric point it is necessary to dilute the gelatin with distilled water of pH 4.7.

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gelatin (pH 4.7) there is an excess of hydrogen ions over the OH ions, there must of necessity be enough diffusible anions to keep the solution electrically neutral. A removal of the last trace of the diffusible ions gives rise to negative protein ions until electrical neutrality is reached. The greater the dilution of the gelatin, the further will the solution be removed from pH 4.7 towards the pH of the distilled water used for dilution.

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COMBINATION OF SALTS AND PROTEINS.

III. THE COMBINATION OF $CuCl_2$, $MgCl_2$, $CaCl_2$, $AlCl_3$, $LaCl_3$, KCl, $AgNO_3$ AND Na_2SO_4 WITH GELATIN.

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The combination of proteins with acids and alkalies has been thoroughly studied by a number of investigators, but the reaction of proteins with salts has received little attention—owing largely to the lack of a convenient method for determining the free ion concentration of those ions for which there is no satisfactory electrode. The problem is of interest since salts have almost as marked an effect on the properties of protein solutions as do acids. It was pointed out in the previous papers[1] of this series that the activity of an ion in a protein solution could be determined by setting up a Donnan equilibrium and measuring the total ion concentration in the pure salt solution outside the membrane, and in the protein solution inside the membrane. If the membrane potential is also measured the ratio of the activity of the ion inside to that outside can be calculated and if the activity coefficients are known then the concentration of the ion inside can be calculated. The difference between this figure and the total concentration found by analysis evidently gives the concentration of combined ion; *i.e.*,

$$M_c = M_t - \frac{M_o \gamma_o}{r \gamma_i}, \tag{1}$$

where M_t is the total concentration of ion found by analysis inside, M_c is the concentration of combined ion, M_o is the total concentration

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 25; 1925–26, ix, 351.

of ion outside and γ_o, γ_i are the activity coefficients of the ion in the outside and inside solutions respectively, and $\log r = .4343 \frac{\text{Membrane Potential}}{RT/nF}$. In the present experiments the total salt concentration differs only slightly between the inside and the outside solution, so that if the effect of the protein on the ionic strength of the solution is neglected[2] then $\gamma_o = \gamma_i$.

Experimental Procedure.

The experiments were carried out as already described except that a more sensitive galvanometer was used. The membrane potentials were measured on four systems and two other systems were analyzed gravimetrically, Cl as AgCl, Cu as CuSCN, Ca as Ca oxalate, Mg as $Mg_2P_2O_7$ and Al as $Al(OH)_3$. Duplicate determinations were made on both the inside and outside solutions of both systems. Experiments in which the analyses differed by more than 0.2 per cent or the potential measurements by more than 0.05 millivolt were not used. The figures given are therefore the averages of 2 analyses of each solution and of four potential measurements. Even under the best conditions, however, the error in the final figure may amount to 10 or 20 per cent, especially in the concentrated salt solutions, since the calculated amount of ion combined depends on a small difference between two large experimental figures.

The isoelectric gelatin was prepared as described in the preceding paper.[4]

Preparation of Deaminized Gelatin.—100 gm. of isoelectric gelatin were dissolved in 1 liter of water and 10 gm. $NaNO_2$ and 15 cc. glacial acetic acid added, then warmed at 80° for 4 hours, at the end of which time all amino nitrogen had been removed.

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[2] As pointed out in the previous paper[1] the experiments give directly the decrease in the activity of the ion in the presence of the protein, but in order to calculate the actual change in concentration of the ion it is necessary to make some assumption as to the effect of the protein on the activity coefficient (*i.e.* on the ionic strength of the solution). In the present calculation it has been assumed that this effect is negligible.

At first sight this appears an improbable assumption since the protein, although present in small concentration, is undoubtedly polyvalent. It may be noted, however, that according to Simms[3] the effective valence of polyvalent ions decreases as the distance between the groups increases, so that gelatin in acid solution has an effective valence of 1.8. It was also shown that the results obtained in this way agree with those obtained for Cl, H^+ and Zn^{++} by means of concentration cells.

[3] Simms, H. S., *J. Gen. Physiol.*, 1927–28, xi, 613.

[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927–28, xi, 477.

TABLE I.

Combination of Copper Chloride and Gelatin.

Concentration of gelatin	Ion determined	P.D.	Concentration of Cu^{++} Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin		
			Inside	Outside	Cu^{++}	Average	Cl^-
per cent		*mv.*					
1.8	Cl	+2.5	10.7	8.8	0.19		0.10
4.5	Cl	8.1	13.1	8.1	.20		.10
			13.2	8.0	.19		
4.5	Cl	5.8	23.8	16.6	.28		.14
4.5	Cl	3.5	54.4	43.95	.44		.18
4.5	Cu	1.8	107.6	94.5	.58		.30
			107.4	91.4	.63	.60	
1.8	Cl	.75	101.3	95.8	.60		.32
1.8	Cu^{++}	.50	185.0	179.0	.72		
			184.0	177.0	.78		
4.5	Cu^{++}	1.1	203.0	187.5	.66	.75 ±.02	.35
			206.2	188.3	.72		
1.8	Cl	.50	199.5	193.0	.78		.28
			199.5	192.0	.84		
4.5	Cu^{++}	.80	355.0	329.0	.98		
			356.0	330.0	.98	.93 ±.03	
1.8	Cu^{++}	.26	342.0	334.0	.83		
1.8	Cl	.15	494.0	484.0	.89		
			497.0	486.0	.92	.90 ±.01	
4.5	Cl	.63	495.2	479.2	.90		.23
			497.5	479.0	.89		.31

The solution was kept at 20° for 26 hours and then dialyzed at 30° in a rocking dialyzing apparatus.[5] Final solution: 6 gm. per 100 cc. deaminized gelatin, specific conductivity 7×10^{-5}: pH (30°) 4.1; H^+ combined at pH 1.51 = 0.43 millimols per gm.

EXPERIMENTAL RESULTS.

Copper Chloride.—Copper is known to form complexes with ammonia and amino groups so that it seemed reasonable to suppose that

TABLE II.

Deaminized Gelatin. 1.7 Per Cent. pH 4.1.

Concentration of gelatin	Ion determined	P.D.	Concentration of Cu^{++} Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin		
			Inside	Outside	Cu^{++}	Average	Cl^-
per cent		*mv.*					
1.7	Cl	.20	194.1	191.8	0.33		0.07
1.7	Cl	.10	293.0	290.5	.31		.2
			295.0	292.0	.34		.25
1.7	Cl	.12	388.0	383.8	.50		.3
			385.0	383.8	.30	.44 ±.03	.27
			388.0	384.0	.50		
			385.0	382.0	.45		
1.5	Cl	.07	491.9	486.5	.49		.40
6.0	Cl	.34	515.0	500.0	.46		
6.6	Cl	.35	723.0	690.0	.50		
5.5	Cl	.35	713.0	708.0	.51		
5.5	Cl	.28	925.0	909.0	.63		
4.3	Cl	.28	930.0	930.0	.47		

it would be found to combine with proteins. The results of the experiments with this salt are shown in Table I. The table shows that the copper ion is combined to a large extent in dilute solution and that on increasing the copper concentration the copper combined per gm. of gelatin increases and reaches a maximum at about 0.9 millimols per gm., which agrees with the equivalent combining power of gelatin for hydrogen ion. In other words, the reaction is stoichiometric

[5] Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1927-28, xi, 641.

since 1 mole of copper is equivalent to 1 mole of hydrogen. Apparently then copper combines with the same groups in the protein molecule as does hydrogen. This conclusion can be verified by determin-

TABLE III.

HCl. $CuCl_2$. Gelatin.
Combination of Hydrogen and Copper.
1.8 Per Cent Gelatin.

pH	P.D.	Final concentration of Cu^{++} Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin		
		Inside	Outside	Cu^{++}	H^+	$Cu^+ + H^+$
	mv.					
4.7	0.70	104.6	96.6	0.70		0.70
		105.5	96.4	.80		.80
4.0	.73	97.3	92.8	.53		
3.0	.53	95.0	96.0	.16	0.71	.87
		94.3	96.3	.11	.71	.82
2.5	.68	96.4	98.3	.17	.72	.89
		96.8	98.1	.20	.69	.89
2.0	.60	95.5	97.8	.12	.85	.97
		94.6	96.9	.12	.85	.97
		4.5 Per Cent Gelatin.				
2.0	8.3	7.1	12.5	.01	.94	.95
2.0	6.6	16.3	23.8	.04	.92	.96
		16.0	23.5	.04	.96	1.00
2.0	3.3	47.6	53.0	.14	.79	.93
		47.3	53.4	.13	.79	.92
2.0	1.9	97.0	101.0	.21	.59	.80
		100.0	100.0	.29	.59	.88

ing the combining power of deaminized gelatin. It was shown by Hitchcock[6] that hydrogen ions reacted stoichiometrically with the

[6] Hitchcock, D. I., *J. Gen. Physiol.*, 1923–24, vi, 95.

nitrogen groups since removing 0.40 millimols of nitrogen per gm. of gelatin decreased the hydrogen equivalent by the same amount.

The experiments were therefore repeated with deaminized gelatin with the results shown in Table II. The combining equivalent is now about 0.4 to 0.5 millimols copper per gm. of gelatin which agrees again with the figure for hydrogen.

In both of the above experiments it is difficult to prove conclusively that the figure obtained is really a maximum value since the experi-

TABLE IV.

$AlCl_3$. 5 Per Cent Gelatin.
Gravimetric Cl Analysis. 37°C.

P.D.	Concentration of Al^{+++}		Millimols combined per gm. gelatin	Millimols Cl combined per gm. gelatin
	Millimols per 1000 gm. H_2O			
	Outside	Inside		
mv.				
25.0	1.41	4.30	0.08	0.037
18.0	3.90	8.75	.16	.065
11.40	8.60	16.0	.27	.16
4.90	30.4	41.0	.47	.24
1.46	107.0*	120.0	.57	.40
1.80	74.3*	86.7	.52	.40
.60	238.0	250.0	.51	.34
.72	276.0	287.0	.62	.16
.50	484.0	495.0	.67	.10
.24	445.7	457.3	.44	.40
.08	993.0	1006.0	.45	.60

*Gravimetric Al^{+++} analysis.

ments cannot be carried into higher salt concentration owing to the experimental errors. If the copper really combines with the same groups as does hydrogen, however, it should be possible to show that the sum of the two ions combined is constant, provided one or the other is present in excess. That is, adding copper to gelatin in the presence of excess acid should result in the displacement of hydrogen ion by copper, while adding acid to gelatin in the presence of excess copper should result in the liberation of copper ions, the sum of the amount of ions combined should however remain constant and equal to 0.9 millimols per liter. The results of the experiments given in Table

III show that this prediction is carried out and confirm the conclusion that the copper and hydrogen combine with the same groups in the protein molecule. The experiments also show that there is an equilibrium between the combined Cu^{++} and H^{+}.

Chloride.—The combined chloride ion in all the experiments is less than the copper and approaches a value of about 0.3 millimols per gm. gelatin in the higher concentrations. The figure with deaminized gelatin is the same as that with gelatin. The chloride therefore combines independently of the copper. Since there is more copper

TABLE V.

$LaCl_3$.

Isoelectric Gelatin. 37°C.

Concentration of gelatin	P.D.	Concentration of La^{+++}				Millimols Cl combined per gm. gelatin
		Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	Average	
		Outside	Inside			
per cent	*mv.*					
10.0	21.6	1.2	4.2	0.04		0.039
10.0	17.85	3.55	8.83	.087		.056
1.8	1.9	10.2	12.1	.21		
1.8	1.10	36.2	39.0	.40		.23
.84	.41	36.3	37.5	.34	0.34	.20
10.0	6.84	25.4	38.7	.27		.17
1.8	.48	111.0	115.7	.57		.37
.85	.20	110.8	112.3	.47	.50	
10.0	3.86	70.1	89.5	.44		.24
10.0	.60	354.0	381.7	.49		.59

than chloride ion combined the resulting protein copper complex should have a positive charge. This was found to be the case, since gelatin in 0.1 M copper chloride migrated to the negative electrode when tested by the U-tube method using non-polarizable electrodes.

Aluminum and Lanthanum.—Loeb[7] found that La^{+++} and Al^{+++} made protein particles positive and suggested that this was due to the formation of a complex ion of the protein and the metal ion. The experiments in Tables IV and V show that both lanthanum and

[7] Loeb, J., Proteins and the theory of colloidal behavior, McGraw-Hill, New York, 1924.

aluminum do combine with gelatin to a considerable extent. The combining equivalent appears to be approaching a value of 0.5 to 0.6 millimols per gm. gelatin but in this case again it is difficult to prove that this is really a maximum value. There seems no doubt, however, that the figure is smaller than that for copper. If the aluminum and lanthanum combined with the other nitrogen groups but not with the NH_2 which are removed with nitrous acid, the combining equivalent would be about 0.5 millimols per gm. and this is the value found. In confirmation of this assumption it was found that the figure for La combined with deaminized gelatin (Table VI) was not significantly different from that for gelatin.

TABLE VI.

Deaminized Gelatin. 37°C.

Concentration of gelatin	P. D.	Concentration of La^{+++}		Millimols combined per gm. gelatin	Millimols Cl combined per gm. gelatin
		Millimols per 1000 gm. H_2O			
		Outside	Inside		
per cent	*mv.*				
1.8	.67	19.91	21.1	.15	.12
		19.95	21.3	.16	.14
1.8	.32	100.2	102.0	.30	
		99.5	101.3	.31	
1.8	.14	302.0	306.7	.54	.44
		295.3	298.7	.45	.22

The chloride combined was nearly the same as that found with copper showing again that the two ions combine independently.

$CaCl_2$.—The results with $CaCl_2$ are given in Table VII. The combined calcium approaches the value of 0.9 millimols per gm. of gelatin as in the case of copper but the maximum is not reached until a much higher concentration of salt. The value with deaminized gelatin at pH 5.0 is the same as for gelatin. Apparently the Ca^{++} therefore does not combine with the NH_2 groups but with the carboxyl groups. The carboxyl group equivalent of gelatin is also about 0.9 millimols per liter.

Magnesium Chloride.—Table VIII gives the results of the experiments with $MgCl_2$. The combined magnesium approaches 0.4 – 0.5 millimols per gm. as in the case of Al and La. This low figure is probably due to the fact that the solutions are slightly acid. The combination with deaminized gelatin is about the same or higher, at

TABLE VII.

$CaCl_2$.

1.6 Per Cent Gelatin. pH 5.0.

P.D.	Concentration of Ca^{++} Millimols per 1000 gm. H_2O		Ca^{++} Millimols combined per gm. gelatin
	Outside	Inside	
mv.			
0.20	296.0	300.8	0.58
	294.0	297.2	.49
.10	496.0	506.0	.78
	492.0	496.0	.47
.10	751.0	758.0	.70
	738.0	744.0	.63
.13	982.0	993.0	1.2
	986.0	996.0	1.1
.36	882.0	914.0	.77
(7.3 per cent gelatin)	916.0	954.0	.83
Deaminized Gelatin pH 5.0 + $Ca(OH)_2$.			
.09	948.0	957.0	1.00
	962.0	968.0	.87
.15	1008.0	1034.0	.90
(4.4 per cent gelatin)	1008.0	1035.0	.89

pH 5.0. $CaCl_2$ also gave low results when the solution was not kept at pH 5.0 by the addition of alkali. In the case of $MgCl_2$ this cannot be done owing to the insolubility of $Mg(OH)_2$.

KCl, NaCl, LiCl.—In all the former experiments the membrane potential has been positive showing that the non-diffusible ion was positive. In the case of KCl however the membrane potential is

negative and it is found that the amount of K combined is within the error of the method. In fact the value comes out very slightly negative. The combined Cl is also less than in the other experiments but is still enough to be significant. The experimental results are shown in Table IX. There is therefore a qualitative difference between KCl

TABLE VIII.

$MgCl_2$.

10 Per Cent Gelatin. 37°C.

P.D.	Concentration of Mg^{++}			Concentration of Cl^-
	Millimols per 1000 gm. H_2O		Millimols combined per gm. gelatin	Millimols combined per gm. gelatin
	Outside	Inside		
mv.				
1.51	15.0	18.0	0.04	0.04
.94	62.0	68.1	.10	.07
.20	569.0	582.0	.21	.14
.30	1027.0	1042.0	.36	.07
.40	1140.0	1151.0	.47	
.30	964.5	982.5	.39	.15
.23	2107.0	2131.0	.60	.12
Deaminized Gelatin. 1.7 Per Cent. pH 4.1.				
.11	303.0	303.8	.18	
	303.0	303.8	.18	
.16	501.5	502.5	.38	−.1
	500.0	501.0	.35	−.2
Deaminized Gelatin at pH 4.7 (+ NaOH).				
.04	298.2	306.0	.49	
	292.8	300.2	.46	
.16	492.0	502.0	.89	
	494.0	504.5	.94	

and the di- and trivalent ions studied, in that KCl forms a negative complex ion while the other salts form a positive complex ion. In any system therefore which requires a very small or no potential there would be a sharp antagonism between the effect of Na, K and Li ions and Mg or Ca ions.

$AgNO_3$.—The results for $AgNO_3$ are given in Table X. As would be expected, Ag^+ is combined more than the other monovalent ions. The nitrate ion was not combined.

Na_2SO_4.—No combination of either ion was found and no potential was obtained.

TABLE IX.

KCl.

10 Per Cent Gelatin. 37°C.

P.D.	Concentration of K		Millimols combined per gm. gelatin	Cl Millimols combined per gm. gelatin
	Millimols per 1000 gm. H_2O			
	Outside	Inside		
mv.				
−0.18	98.0	96.8	−0.02	0.1
	98.3	98.8	− .02	.1
− .05	499.4	497.8	− .02	
	497.8	496.8	− .01	.05

TABLE X.

$AgNO_3$.

5 Per Cent Gelation. 37°C.

P.D.	Ag^+		Millimols combined per gm. gelatin	NO_3^- Millimols combined per gm. gelatin
	Millimols per 1000 gm. H_2O			
	Outside	Inside		
mv.				
3.12	17.4	20.9	0.10	0.02

TABLE XI.

Na_2SO_4. 1.8 Per Cent Gelatin.

pH	P.D.	Concentration of $SO_4^=$ Millimols per 1000 gm. H_2O	
		Outside	Inside
	mv.		
4.7	0.2	96.8	96.9

Effect of the Hydrogen Ion Concentration.—The foregoing experiments were all done as nearly as possible at pH 4.7, with the exception of those in which acid was added to the copper. It was found in that case that the copper was displaced by the addition of acid. In the case of copper it is not possible to work on the alkaline side so that it seemed of interest to complete the experiment with another ion.

The results of a series of experiments with 0.1 M $CaCl_2$ at various pH values are given in Table XII. As in the case of copper the addition of acid quickly replaces the calcium so that on the acid side of

TABLE XII.

HCl. $CaCl_2$.
1.8 Per Cent Gelatin.

pH	P.D.	Concentration of Ca^{++} Millimols per 1000 gm. H_2O		Ca^{++} Millimols combined per gm. gelatin
		Outside	Inside	
	mv.			
2.5	0.50	100.0	96.2	
3.4	.3	101.5	99.0	
3.8	.3	98.6	98.2	0.10
4.1	.5	100.0	99.5	.19
4.7	.4	98.0	100.0	.29
5.4	.10	96.5	98.5	.15
7.0	.2	100.6	103.8	.29
8.6	.12	98.8	102.9	.26
10.0	.24	99.4	105.0	.35
		99.7	104.4	.41

pH 3 no combined calcium was found. The value rises rapidly as the solution becomes less acid and remains approximately constant on the alkaline side of pH 4.7.

SUMMARY.

1. The combination of Cu^{++}, Ca^{++}, Mg^{++}, Al^{+++}, La^{+++}, K^+, Ag^+, and Cl^- with gelatin has been determined.

2. The equivalent combining value for copper is about 0.9 millimols per gm. of gelatin and is therefore the same as that of hydrogen. The value for copper with deaminized gelatin is about 0.4 to 0.5, again the

same as that of hydrogen. The sum of the hydrogen and copper ions combined in the presence of an excess of either is 0.9 millimols showing that there is an equilibrium between the copper hydrogen and gelatin and that the copper and hydrogen are attached to the same group.

3. The equivalent combining value of La^{+++} and Al^{+++} is about 0.5 millimols per gm. of gelatin. This value is not significantly different with deaminized gelatin so that it is possible these salts combine only with groups not affected by deaminization.

4. No calcium is combined on the acid side of pH 3. The value rises rapidly from pH 3 to 4.7 and then remains constant.

5. No combination of K, Li, Na, NO_3 or SO_4 could be detected.

6. Cl combines less than the di- and trivalent metals so that the protein is positive in $CaCl_2$ but negative in KCl.

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DIALYSIS WITH STIRRING.

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(Accepted for publication, December 22, 1927.)

The separation of the diffusible from the non-diffusible substances by dialysis through a collodion membrane is a slow process when the solution remains stagnant even though fresh water be circulated on the other side of the membrane. This has led to the adoption of electrodialysis, which has proved successful in some cases, but which has the disadvantage of complexity and of electrolysis.

In order to hasten diffusion, Northrop and Kunitz[1] adopted the method of placing toy glass "marbles" in their collodion bags. The bags were immersed in water in glass tubes which were rocked mechanically, causing the marbles to roll from end to end of the bags. This method of stirring has been slightly modified for the purification of proteins. The apparatus is shown in Fig. 1. Collodion bags are made as described by Northrop and Kunitz.[1] These are placed in the tubes and distilled water of any desired temperature is allowed to circulate past them while they are rocked by means of an automobile windshield scraper.

The effect of stirring was determined by filling two membranes with 0.5 M NaCl and measuring the rate of diffusion with a flow of distilled water of 8 cc. per tube per minute. One membrane was rocked, the other was stationary. The results are given in Table I.

A thick suspension of egg albumin crystals in concentrated ammonium sulfate was dialyzed in a similar apparatus placed in a cold bath (5°C.) with a flow of 10 cc. distilled water per tube per minute. The results are given in Table II.

The completeness of the dialysis is shown by the specific conductivity of 8.2×10^{-5} reciprocal ohms after 48 hours. (The protein concen-

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925-26, ix, 351.

tration was 14 per cent by the dry weight method.) A second batch gave 2.4×10^{-5} after 96 hours dialysis with shaking (17.1 per cent protein). This compares favorably with the value of 2.1×10^{-5} obtained by Pauli[2] (on 4 per cent albumin) after long dialysis followed by electrodialysis. The value obtained by Svedberg and Nichols[3] on egg albumin which was dialyzed in flowing distilled water for 18 days and subsequently electrodialyzed for about 2 days, was 3.42×10^{-5} for a 6.6 per cent solution.

TABLE I.

Effect of Stirring on Diffusion of 0.5 M NaCl.

Time of dialysis	Specific conductivity × 1,800	
	Stirred	Not stirred
hrs.		
0	100	100
2	5	27
4	0.3	9
6	0.1	1.4

TABLE II.

Dialysis of Egg Albumin in Concentrated Ammonium Sulfate, While Stirring.

Time of dialysis	Specific conductivity × 10^{-5}
hrs.	
4	820
7	210
24	18
31	16
48	8

Deaminized gelatin containing a large amount of electrolyte was dialyzed to 6.4×10^{-5} reciprocal ohms (6.7 per cent protein) in 24 hours in this apparatus.

Description of Apparatus.

The apparatus is shown in Fig. 1 and does not require detailed description. Distilled water is supplied by a bath which may be kept

[2] Pauli, W., *Biochem. Z.*, 1925, clxiv, 400.

[3] Svedberg, T., and Nichols, J. B., *J. Am. Chem. Soc.*, 1926, xlviii, 3081.

at higher or lower or at constant temperature as desired. The water siphons down the inlet tube into the three glass tubes each containing four stoppered collodion sacs filled with the solution and containing a glass marble. Each sac holds 30 to 35 cc. of solution. The water then flows out the outlet tube. The flow of water is regulated with a screw clamp.

In the case of solutions not affected by air, a large bubble of air may be left in the bags in place of the marbles.

The glass tubes are attached with copper wire to a board which hinges on two screws and is rocked by means of a windshield scraper.

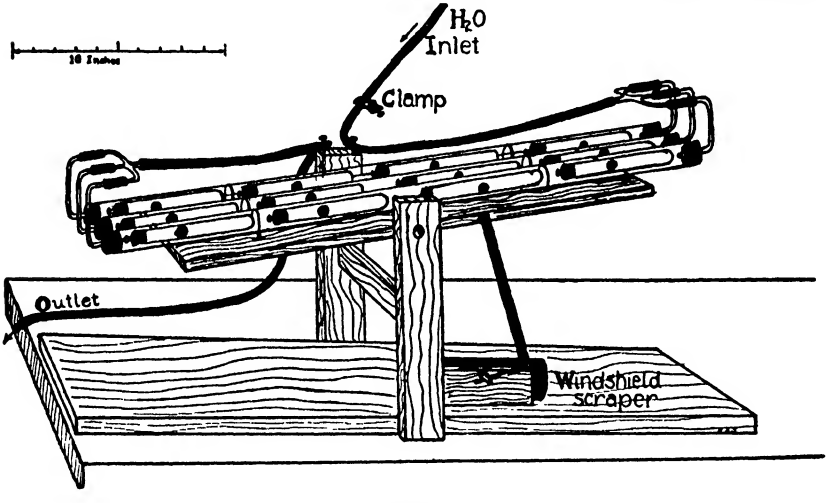

FIG. 1.

The rate of rocking is adjusted to a speed such that the marbles pass from one end of the sacs to the other (*i.e.*, 1 cycle in about 6 seconds).

The rate of flow of water is about 8 cc. per tube per minute. If the concentration of dialysate is high 2 to 3 times as much water should be used for the first 2 to 3 hours. For running overnight a large amount of water is required. If a wash-boiler or other tank is not enough, two or more 5 gallon bottles (on the same level) may be connected to it with large siphon tubes.

The windshield scraper is better adapted to most laboratory uses if the axis of the arm is turned 90° from its original position. This may be done by removing the shaft and filing or drilling it to make a seat for the set screw 90° from the original position.

SUMMARY.

Substances to be purified by dialysis are placed in collodion bags together with a toy "marble" or a bubble of air. The bags are stoppered and placed in glass tubes of a rocking machine. Distilled water of the desired temperature is circulated through the tubes (around the bags) at a rate of about 8 cc. per minute per bag while the machine is in motion. The rolling of the marbles or bubbles causes stirring which makes it possible to remove the salts from a protein solution in 24 to 48 hours.

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POSITIVE AND NEGATIVE CURRENTS OF INJURY IN RELATION TO PROTOPLASMIC STRUCTURE.*

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Much may be learned about the nature of protoplasm by studying its transformations during the process of death. Such alterations have been observed by a method which has marked advantages since it permits us to follow very rapid changes which are often of primary importance. It may be added that certain complications inherent in tissues have been avoided by using single multinucleate cells.

The outcome, which supports the idea that protoplasm is made up of layers differing considerably in their properties, appears to necessitate a change in the traditional view that the current of injury is negative[1] for the observations show that it can be rendered positive or negative according to the will of the experimenter.

The experiments described in this paper were performed on single multinucleate cells of *Nitella flexilis*[2] according to the methods given in a previous paper[3] (which contains a description of the technique and of the apparatus). A flowing contact was used in most cases. Especial care was taken to employ only normal cells, and to avoid injury (except in applying toxic agents). The current of injury was produced by chloroform which has previously been found useful for experi-

* The writers desire to express their gratitude to the Carnegie Institution of Washington, D. C., which generously provided for the beginning of these researches in 1922–25.

[1] A discussion of the literature will be deferred to a later paper dealing with cell groups and tissues. It may be mentioned that a few experiments on the effect of chloroform on *Nitella* were performed by Haacke (Haacke, O., *Flora*, 1892, lxxv, 455). Hörmann (Hörmann, G., Studien über die Protoplasmaströmung bei den Characeen, Jena, 1898) states that stimulation of one end of a *Nitella* cell produces a wave of negativity.

[2] Kindly identified by Dr. J. S. Karling.

[3] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391.

ments on injury.[4] The experiments were carried out at room temperature, ranging from 20° to 25°C. but the variation in any one experiment was less than 1°C.

The procedure may be illustrated by an experiment with 0.05 M KCl[5] in contact with the cell at A and C (as shown in Fig. 1). Fig.

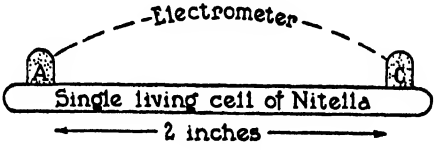

FIG. 1. Diagram to show the arrangement of the experiments. In most cases flowing contacts are employed at A and C. In some cases the ends of the cell dipped into two cups hollowed out of a block of paraffin: in experiments with sap cotton soaked in sap is applied. When necessary (with more concentrated solutions) cotton soaked in distilled water is applied for a short stretch between A and C but a space is left on each side of the cotton to prevent short circuiting.

2 shows at the start a small potential difference[6] between A and C.[7] We now substitute for 0.05 M KCl at C a solution of 0.05 M KCl saturated with chloroform. This injures the protoplasm at C and produces characteristic changes in the curve but it subsequently reaches a steady state which is assumed to mean that the protoplasm

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 709. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391. Chloroform is soluble to the extent of about 0.4 per cent by volume (about 0.6 per cent by weight) and hence does not noticeably affect the osmotic pressure or concentration of electrolytes.

[5] This gives practically the same result as sap or artificial sap. It would appear that this result must be due primarily to the effect of the cations since the halide content of sap is about the same as that of 0.1 M KCl, but the K content of sap is much less.

[6] To avoid misunderstanding we may state that we use the term electromotive force to designate the force present at an electrode surface: if we tap off at two points in the circuit we obtain a potential difference which depends on the resistances involved.

[7] The recorded values would probably be a trifle higher if we could eliminate short-circuiting effects in the cell (Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83). Diffusion potentials are small enough to be neglected (in view of the concentration of KCl present).

at C is dead.[8] For reasons given in a previous paper[3] we now regard the potential difference as due entirely to the living protoplasm at A. We now apply 0.05 M KCl saturated with chloroform to A and obtain a curve similar to that already observed except that the signs are reversed. This is to be expected since the curve always records the state of A with reference to C and any changes occurring at C will have their signs reversed: hence to determine the effect of chloroform on the protoplasm we must take the latter part of the curve (when the chloroform is applied to A) where the signs have their true value (or we may take the first part of the curve and reverse the signs).

We see that when A is injured by applying chloroform dissolved in 0.05 M KCl A becomes more positive[9] with reference to C. Since the

[8] The use of a flowing contact[3] largely eliminates disturbances due to the coming out of sap immediately after the death of C. If the flowing contact were not employed we should not have the same salt solutions in the cell wall at A and C and in consequence there might be a potential difference (sap coming out at C earlier than at A would tend to make the cell wall at C more negative so that on the record A would appear more positive). The flowing contact promptly washes the sap out of the cell wall and thus removes this source of disturbance. As an example of this we may consider the following experiment: Live cells were placed in pure chloroform (not in an aqueous solution of chloroform) which quickly kills them without allowing salts to escape from the cells. One end of the cell was then cut and the protoplasm and sap squeezed out for a distance of an inch so that the lumen was filled with air and could not supply salts to the cell wall any more than the living protoplasm would. We will call this end A and the other C. Both A and C were alike in having the cell wall imbibed with sap, due to the death of the protoplasm (which is not quite the same thing as applying sap from the outside, as will be discussed in a later paper), but they differ in that C has a reservoir of sap in the lumen of the cell which A has not. On leading off from A to C, with 0.05 M KCl applied to each, there was practically no potential difference but when 0.001 M KCl (flowing junction) was applied to A, it quickly became positive showing that the salts were being rapidly washed out. When the value became constant, 0.001 M KCl (flowing junction) was applied at C and the potential difference rapidly fell to zero showing that the salts were washed out of the cell wall despite the reservoir of sap in the lumen of the cell.

The flowing contact did not produce complications except possibly in very dilute solutions and if disturbances occurred in experiments of any sort (as shown by the fact that the curve did not fall to zero when the cell was killed) the experiments were rejected.

[9] In all cases we obtain essentially the same result when we apply a toxic agent at A whether we have previously killed C or not. The only difference is that if C

protoplasm at C is dead we may regard its electromotive force as approximately zero[3] so that all the potential differences observed are

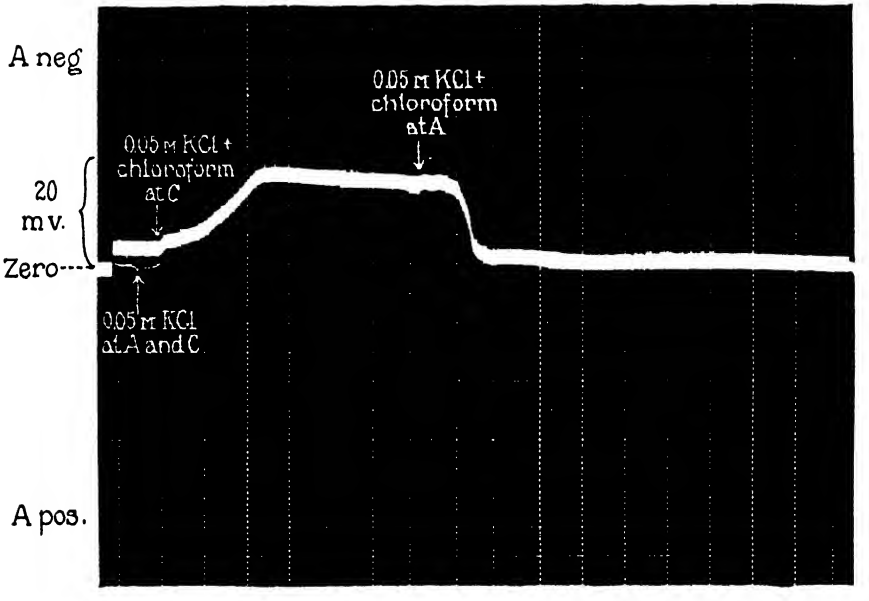

FIG. 2. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.05 M KCl at A and C. When 0.05 M KCl saturated with chloroform is applied at C the curve (which records the potential difference of A with reference to C) shows that A appears to become more negative but since it is only C which changes, the curve really shows that C is becoming more positive. The potential difference between A and C soon reaches a fixed value which is practically all due to the living protoplasm at A. A solution of 0.05 M KCl saturated with chloroform is then applied at A, giving a death curve which resembles that already observed but with opposite signs since the signs now have their true value (C being dead remains constant). The curve shows that under these conditions injury causes the protoplasm to become more positive after which the potential difference approaches zero.

The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

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remains alive it adds a certain constant (positive or negative) value (due to the presence of the living protoplasm at C): in consequence the curve of A does not approach zero at the end but reaches a steady state at a definite positive or negative value which represents the potential difference across the protoplasm at C.

due to A which, as shown in a previous paper,[8] can apparently remain normal for some time after the death of C.

It will be noticed that there is no trace of the traditional negative current of injury. The observed change in potential difference caused by the toxic agent is wholly in the positive direction.

It may simplify matters if before describing other experiments we present an hypothesis which brings the observed phenomena under a single point of view and which may thus facilitate the subsequent treatment.

According to this hypothesis the protoplasm has an outer (X) and an inner (Y) surface layer, both of which are probably non-aqueous,

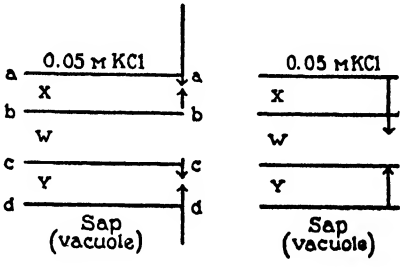

FIG. 3 *a*. FIG 3 *b*.

FIG. 3. Hypothetical diagram to illustrate the condition of the protoplasm in contact with 0.05 M KCl. The direction in which the positive current tends to flow is shown by the direction of the arrows, the relative magnitude of the electromotive force being indicated by their length. Fig. 3 *a* represents a possible conception of the potential differences; Fig. 3 *b* presents the same thing in simplified form. The potential difference across the protoplasm is said to be negative since the negative arrow at X is longer and the positive current tends to flow as indicated in Fig. 4 *b*.

with an aqueous layer, W, between them. On this basis we may diagram the potential differences in the protoplasm in the manner shown in Fig. 3 *a* and 3 *b*, the arrows representing the direction in which the positive current tends to flow and their length indicating the relative magnitude of the potential difference.[10] The underlying theory of these effects has been discussed in a previous paper[11] and

[10] The arrows are hypothetical illustrations and their lengths can be varied accordingly.

[11] Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83.

the conclusion reached that in general the cations have a greater tendency to enter X and Y than the anions. Hence we may suppose that at the surfaces a, b, c and d (Fig. 3 a) the potential differences may be represented by the arrows if they are due to phase boundary potentials. Since we are merely concerned with the resultant of these

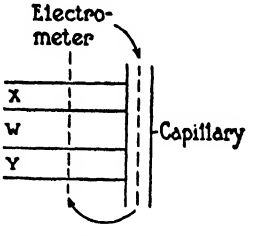

FIG. 4 a.

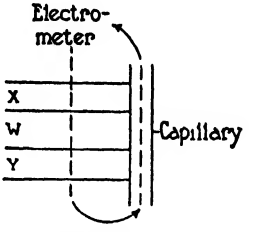

FIG. 4 b.

FIG. 4 a. Diagram of a circuit through the protoplasm at a single spot where an imaginary capillary has been inserted so as to lead off from the outside of the protoplasm to the inside. In this case the potential difference across the protoplasm is said to be positive because the electrometer shows X to be positive to Y.

FIG. 4 b. As in Fig. 4 a but in this case the potential difference across the protoplasm is said to be negative because the electrometer shows X to be negative to Y.

effects it is more convenient to give the diagram the simpler form shown in Fig. 3 b in which only two arrows[12] are employed: this would also be appropriate if diffusion potentials[13] play a part.[14]

In the diagram in Fig. 3 b the arrow at X is longer than that at Y indicating that the positive current tends to flow as in Fig. 4 b. Hence

[12] The actual length of the arrows is not significant: it is merely intended to show that the X arrow is longer than the Y arrow.

[13] Such diffusion potentials might well be due to organic substances produced by the cell (*e.g.*, in the layer W). It is commonly said that diffusion potentials cannot produce potential differences of the magnitude found in living cells. This would be true of aqueous solutions but in non-aqueous media or in such structures as the collodion membranes studied by Michaelis and his coworkers higher values may be possible.

[14] Whether we suppose that the potential difference results chiefly from diffusion potentials due to substances produced in W or to phase boundary potentials (following the scheme in Fig. 3 a) it seems highly probable that the arrows in Fig. 3 b should point in opposite directions.

if we arrange an experiment as in Fig. 1 with 0.05 M KCl at A and C and lead off from A to C after C has been killed by chloroform (thus reducing its potential difference approximately to zero) we should expect the positive current to flow from C to A so that A would appear negative. This is actually the case, as is evident from Fig. 2.

Although little is known regarding electrical conditions in the protoplasm it may be of interest to consider certain possibilities which would exist if the cell conformed to the diagram in Fig. 5. Here we confine ourselves to the assumption that there is but one seat of electromotive force in X and one in Y (but they may exist at both surfaces of any

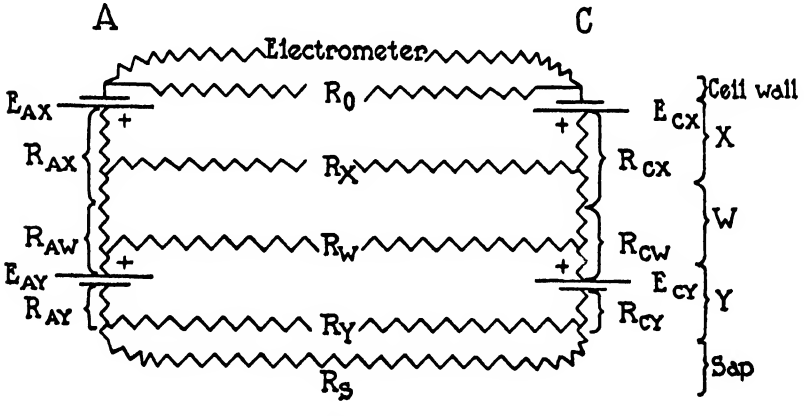

FIG. 5. Hypothetical diagram of electrical conditions in the protoplasm. For the sake of simplicity but one source of electromotive force is assigned to X and one to Y (those in the cell wall and W are regarded as negligible). The resistance in Y is regarded as having vertical components (R_{AY} and R_{CY}) and a horizontal component (R_Y): this also applies to W and X.

layer and possibly in the interior, due to diffusion potential) and we have arbitrarily located this at the outer surface in each case. On this basis the total electromotive force may be regarded as $E_{AY} - E_{AX} - E_{CY} + E_{CX}$.

The resistance in each layer may be regarded as consisting of a vertical component (such as R_{AY}) and a horizontal component (such as R_Y). The resistances R_X and R_Y may be relatively high: R_W and R_S are probably less. As to the resistance R_0 it may be said that the measurements of Dr. Blinks show that when we cut out a portion of a

cell about an inch in length, remove the protoplasm and sap by squeezing, rinse in tap water, squeeze the contents out and dry the surface lightly with filter paper, the resistance is in the neighborhood of a megohm. That of a similar length of intact cell is about half as much which might indicate that under these conditions the resistance of the living protoplasm and sap taken together is not far from that of the cell wall.[15]

But, according to Dr. Blinks, it seems possible that the resistance due to the living protoplasm and sap taken together (which we may call R_P) may be largely due to polarization: hence if measured with currents as small as those present in our ordinary experiments R^P might be quite small in comparison with R_O. In that case we might write

$$\text{observed P.D.} = \text{E. M. F. in circuit} \left(\frac{R_O}{R_O + R_P}\right)$$

and the value of $\frac{R_O}{R_O + R_P}$ would be not far from 1 so that the measurements described in this paper would not be far from the true values of the electromotive forces in the circuit. That this is actually the case is indicated by the measurements obtained when a capillary is inserted in the manner employed for *Valonia*.[11]

Let us now consider what would happen if we had a simpler system from which the layers X and W were omitted. We should then be measuring by means of the electrometer the potential difference across the ends of R_O and we should have

$$\text{observed P.D.} = E_{AY} - E_{CY}\left(\frac{R_O}{R_O + R_{AY} + R_{CY} + R_{YS}}\right)$$

in which R_{YS} is the combined resistance of R_Y and R_S (and is equal to $(R_Y)\ (R_S) \div R_Y + R_S$).

The application of chloroform to C would presumably do away

[15] If we call the combined resistance of the living protoplasm and the sap R_P we might employ the usual equation for resistances in parallel and regard the effective resistance of the living cell as equal to $(R_P)\ (R_O) \div R_P + R_O$. If we have $R_P = R_O = 1$ megohm the effective resistance will be equal to 0.5 megohm.

with E_{CY} and reduce R_{CY} to negligible proportions so that the remaining potential difference would be

$$\text{observed P.D.} = E_{AY}\left(\frac{R_O}{R_O + R_{AY} + R_{YS}}\right).$$

When we speak of the potential difference across the protoplasm at A[16] (after C has been killed) it seems by no means impossible that we must understand it as conforming to a scheme which is similar to this but one which involves all the electromotive forces and resistances in the cell (including any possible eddy currents, as discussed in a previous paper[11]).

Future investigation must decide to what extent the conditions in the protoplasm are represented by such a scheme as that shown in Fig. 5. It is, of course, evident that the cell wall, X, W and Y are not actually insulated from each other as they are in the diagram nor are the electromotive forces confined to A and C.

We may suppose that if we have a simple system such as is represented by the diagram in Fig. 5 the electromotive force after the killing of C is

$$\text{E.M.F.} = E_{AY} - E_{AX}$$

of which we measure by means of the electrometer only a certain fraction so that we may write

$$\text{observed P.D.} = n(E_{AY} - E_{AX}) = n(E_{AY}) - n(E_{AX}).$$

If we put $y = n(E_{AY})$ and $x = n(E_{AX})$ we may write

$$\text{observed P.D.} = y - x.$$

[16] The observed P.D. does not show much change when we shorten the distance between A and C (both being intact) so that we may regard the observed potential difference as practically that of the area directly under A. This fact indicates that R_O cannot be small in comparison with $R_{AY} + R_{CY}$ for if it were the shortening of the distance between A and C would greatly diminish the observed potential difference since the value of $R_{AY} + R_{CY}$ would remain unchanged.

It is evident that we cannot arrive at the true value of the electromotive force at A (after killing C) unless we know the value of the fraction found by dividing R_O by the other resistances properly combined (measurements with an inserted capillary indicate that this value is not far from 1).

If we could destroy X without altering W or Y it is evident that both E_{AX} and R_{AX} would disappear and that this would not only change the electromotive force but would also alter the value of n. In view of this and possibly other complications (such as the presence

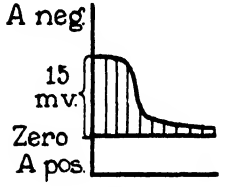

FIG. 6. *a*.

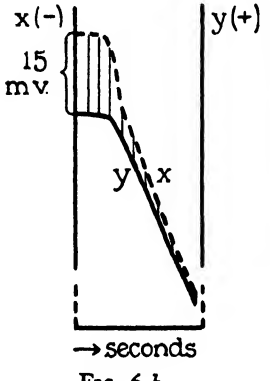

FIG. 6 *b*.

FIG. 6 *a*. Tracing of the latter part of the curve shown in Fig. 2 (after C has been killed by chloroform so that the entire electromotive force is due to the living protoplasm at A). The curve shows that at the start the value of A is negative (-15 millivolts). After the application of chloroform at A it becomes more positive since the potential difference diminishes and approaches zero. Certain ordinates are shaded for comparison with Fig. 6 *b*.

FIG. 6 *b*. Theoretical interpretation of Fig. 6 *a*. The ordinates as drawn represent the observed potential difference which is equal to that of Fig. 6 *a*. Fig. 6 *a* shows only the observed potential difference but Fig. 6 *b* indicates that this is equal to the difference between x with negative sign (as shown by the scale of ordinates at the left) and the value of y with positive sign (as shown by the scale of ordinates on the right). Each ordinate in Fig. 6 *b* has the same length as the one directly above it in Fig. 6 *a*, and the distance between x and y is equal to the distance from zero of the curve in Fig. 6 *a*. Since x and y are purely hypothetical curves they may be drawn in a variety of ways but the most natural assumption seems to be that they have a simple and regular form and they have been drawn in conformity with this idea.

Since we do not know the absolute value of the ordinates in Fig. 6 *b* but only the value of the difference between x and y (*i.e.*, the value of the ordinates as drawn) the scales of ordinates are interrupted toward the base by a dotted line to signify that below this point the ordinate extends for an indefinite distance. It should be noted that if the x and y curves come together the observed potential difference is zero but this zero has no relation to the absolute zero of Fig. 6 *b*.

of more sources of electromotive force, eddy currents,[11] etc.) we shall employ the equation P.D. $= y - x$ merely for convenience in discussion and only in a qualitative sense, meaning that in a general way the observed potential difference depends on a factor due to X and on one due to Y.

Let us now consider the mechanism of the death process. Since chloroform penetrates with extreme rapidity (often killing in a few seconds) and since the protoplasm is only a few microns thick it seems probable that Y will be affected almost as soon as X. If X and Y are in contact with the same solution (sap) or with solutions which have been found[17] to act alike (*e.g.* if X is in contact with 0.05 M KCl and Y in contact with sap) we may expect both to change at the same rate if both are equally sensitive. This is sometimes observed and we might in that case interpret the result in the manner shown in Fig. 6 *b*, since it seems reasonable to suppose that when chloroform acts on X and Y the values of x and y may fall off in regular fashion, as is observed in the death curves of tissues[18] and of single cells (as shown by the unpublished experiments of Dr. Blinks). This assumption is by no means necessary but we may adopt it as the most natural and as a matter of fact it suffices to explain all the observed curves. If, for example, the application of chloroform + 0.05 M KCl causes x and y to fall off in some such fashion as that shown in Fig. 6 *b* it is evident that if we plot the difference between them ($y - x$)[19] in the usual way we shall get the curve shown in Fig. 6 *a* which is a tracing of the latter part of the curve in Fig. 2.

We do not know the absolute value of the ordinates in Fig. 6 *b* but only the difference between them. Hence the scales of ordinates are interrupted toward the base by dotted lines to signify that below this point the ordinates extend for an indefinite distance. It should be noted that when the x and y curves come together, the observed potential difference (as in Figs. 2 and 6 *a*) is zero, but this zero has no relation to the absolute zero of Fig. 6 *b*.

The nature of the change produced by chloroform is uncertain. It

[17] This is found by applying sap at A and 0.05 M KCl at C when it is observed that both act alike in that the potential difference between A and C is zero. We obtain the same death curve with sap saturated with chloroform as with 0.05 M KCl saturated with chloroform.

[18] *Cf.* Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Philadelphia, 1922; *J. Gen. Physiol.*, 1922–23, v, 709. *Cf.* Klopp, J. W., *J. Gen. Physiol.*, 1924–25, vii, 39.

[19] Since the value of A is negative the ordinates of x (which is negative by convention) are made greater than those of y (which is positive by convention).

may cause a loss both of electromotive force and of resistance.[20] It is quite possible that the first effect of chloroform may be to produce in X and Y very minute openings or small electrical leaks (some of which may be almost instantly repaired). This might have a certain

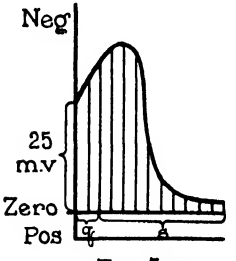

FIG. 7 *a*.

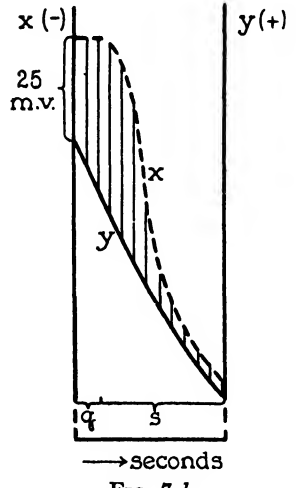

FIG. 7 *b*.

FIG. 7 *a*. Like Fig. 6 *a* but showing a different result. This is a tracing of the latter part of the curve in Fig. 8 (after the counter movement).

FIG. 7 *b*. Theoretical interpretation of Fig. 7 *a*. The ordinates as drawn show the observed potential difference (which is equal to that of Fig. 7 *a*). Fig. 7 *a* shows only the observed potential difference but Fig. 7 *b* indicates that this is equal to the difference between the value of x (with negative sign) and that of y (with positive sign). Each ordinate has the same length as the one directly above it in Fig. 7 *a*. During the period marked q the value of x remains stationary while that of y falls off corresponding to the fact that the curve in Fig. 7 *a* becomes more negative. During the period marked s the two curves in Fig. 7 *b* approach each other as the curve in Fig. 7 *a* approaches zero; if they should meet the potential difference would be zero (but this zero has no relation to the absolute zero of Fig. 7 *b*). *Cf.* Fig. 6. *b*.

similarity to the effects of mechanical injury. The fact that X and Y are changed so readily by chloroform and other lipoid solvents might suggest that they are lipoid: they are also very susceptible to

[20] In the case of *Laminaria* chloroform, ether, and alcohol cause a temporary increase of resistance (Osterhout, W. J. V., Injury, recovery, and death, Philadelphia, 1922) but this is not true of all organisms and may not be the case with *Nitella*. The increase of resistance is followed by a decrease which continues until death ensues: during this period recovery (partial or complete) is possible in the case of alcohol and to a very much smaller extent in the case of ether and chloroform.

the action of acid, alkali, concentrated salt solutions, and mechanical injury, but this might not be inconsistent with the assumption that they are lipoid in nature. We must also consider the possibility that they may act like proteins, agar, starch paste or kaolin.[21]

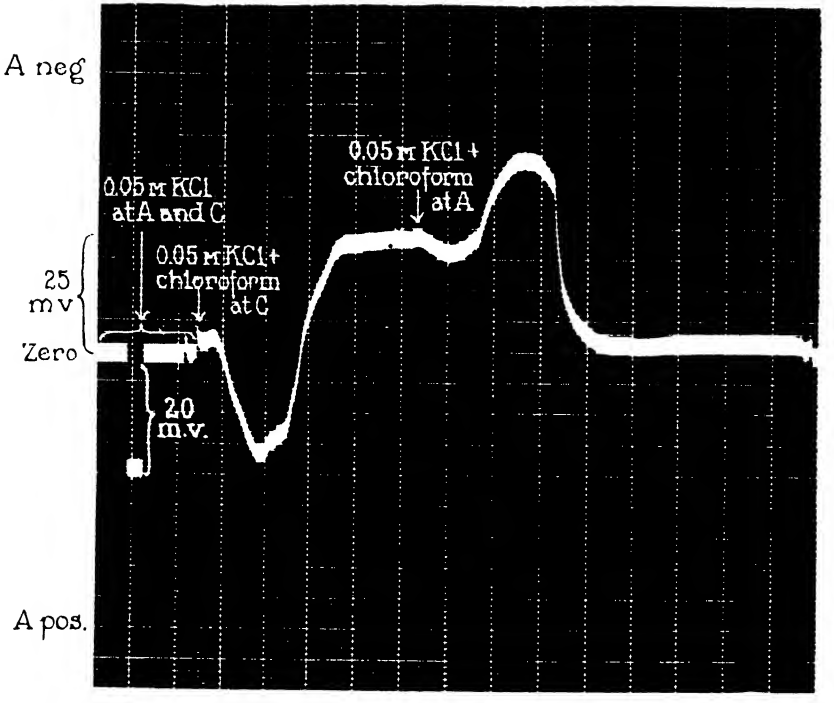

FIG. 8. Like Fig. 2 but showing a different result. After the counter movement following the application of chloroform at *A* (when the signs have their true value) we see that *A* becomes more negative, then more positive: the potential difference then sinks almost to zero. The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

The fact that alcohol produces effects similar to those of chloroform and that they are readily reversible up to a certain point suggests the possibility that the loss of potential difference may be due to an

[21] *Cf.* Höber, R., *Z. physik. Chem.*, 1924, cx, 142. Matsuo, T., *Arch. ges. Physiol.*, 1923, cc, 132. Mond, R., *Arch. ges. Physiol.*, 1924, cciii, 247. Fujita, A., *Biochem. Z.*, 1925, clxii, 245. Deutsch, W., *Arch. ges. Physiol.*, 1925, ccix, 675. Beutner, R., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 462.

increase in the conductivity of X and Y rather than to the production of leaks (there is no reason to suppose that the latter process would be irreversible from the start).

It is also possible that the falling off of potential difference may be due to a loss of electromotive force (independent of or combined with a change in resistance). If the electromotive force is due to unequal mobilities of the ions or unequal partition coefficients it is quite possible that any substance which goes into solution in X and Y could induce alterations in this respect (reversible or irreversible).

In the case of Fig. 2 we may suppose that X and Y change simultaneously but this need not be a general rule. If Y should go a little before X we might get curves similar to those in Fig. 7 *b*, corresponding to the curve shown in Fig. 7 *a*, which is a tracing of the latter part of the photographic record in Fig. 8. As a matter of fact such curves are much more common than the type shown in Fig. 2.

In Fig. 8 and some of the other figures the first large movement after the application of chloroform is preceded by a small movement in the opposite direction. This will be called the counter movement. It might be explained as due to the fact that the chloroform strikes X first so that we might expect the change in X to begin a little before that of Y. Since the "counter movement" is not a constant feature and may possibly be explained in other ways it will be omitted from the theoretical diagrams in this paper.

The fact that Y is apt to change more rapidly than X might be due to differences in the layers themselves or to the solutions in contact with them. The latter seems improbable since we find the same thing when we replace 0.05 M KCl by sap or artificial sap. We may regard all of these solutions as equally "effective" in this respect (we shall use the word "effective" in this special sense throughout the subsequent discussion, the most "effective" solution being the one which most assists the action of the chloroform in changing the potential difference). On the other hand, we know from previous experiments[3] that the two layers are not identical in their properties and we may therefore assume that Y is more sensitive than X to the action of chloroform.

Let us now consider an experiment with a more dilute solution,

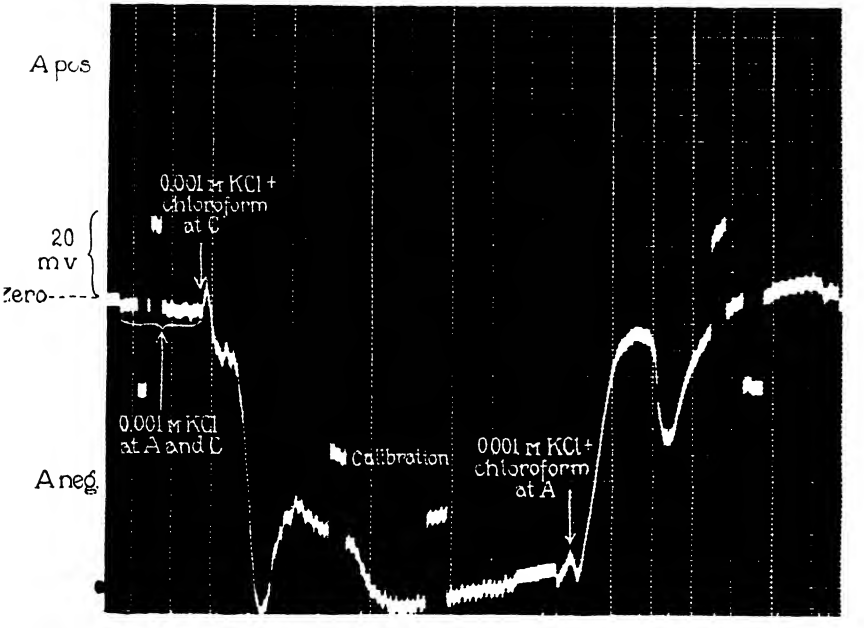

FIG. 9. Photographic record of potential differences; the experiment is arranged as in Fig. 1: at the start 0.001 M KCl is applied at *A* and *C*. After the counter movement which follows the application of 0.001 M KCl saturated with chloroform at *A* we see that *A* becomes more negative, then more positive and then approaches zero (*C* being dead remains constant).

The first part of the curve (resulting from the application of 0.001 M KCl saturated with chloroform to *C*) is similar but with signs reversed because the changes are occurring at *C*: after the death of *C* the curve reaches a fixed value which is practically all due to the living protoplasm at *A*. Chloroform is then applied at *A* giving the death curve already described.

The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

Calibration marks are shown at four places. Small irregular fluctuations are due to movements of the operator, mechanical disturbances or sound waves: they are more noticeable with dilute solutions which introduce greater resistance in series with the living cell.

namely 0.001 M KCl: the result is shown in Fig. 9. The curve shows that after *C* is dead *A* is positive:[22] hence we diagram the protoplasm

[22] This is in harmony with the results of unpublished experiments (made by us in 1922) which show that dilute solutions are positive to more concentrated solutions, as seems to be usually the case with uninjured living cells.

as in Fig. 10 with the arrow at Y^{23} longer than that at X. After the counter movement the first effect of chloroform is to make A more negative;[9] hence we infer that Y changes more rapidly than X. This might be expected on the ground that it is in contact with the more concentrated solution and it appears that in general, even in the absence of chloroform, concentrated solutions render the protoplasm unstable and more easily injured. We therefore regard sap as a more effective solution than 0.001 M KCl.

In order to account for this result we may refer to the diagram in Fig. 11 *b*. We draw hypothetical curves for x and y (just as in Figs. 6 *a* and *b* and 7 *a* and *b*) in such a manner that the values of $y - x$ when plotted as in Fig. 11 *a* give us the latter part of the curve in Fig. 9 (of which the curve in Fig. 11 *a* is a tracing).

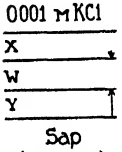

FIG. 10.

FIG. 10. Hypothetical diagram to illustrate the condition of the protoplasm in contact with 0.001 M KCl. The direction in which the positive current tends to flow is shown by the direction of the arrows, the relative magnitude of the electromotive force being indicated by their length. The potential difference across the protoplasm is said to be positive since the positive current tends to flow as in Fig. 4 *a* (since the positive arrow at Y is longer).

The variations which are observed are such as the hypothesis might lead us to expect. For example if the process in X were a little slower we should get the situation shown in Fig. 12 *b*, which would give the observed curve shown in Fig. 12 *a*. We should also expect that the y curve might sometimes drop for a time below the x curve in the latter part of its course making the potential difference temporarily negative and such cases are actually observed.

Let us now consider an intermediate concentration (0.01 M KCl): this is illustrated by Fig. 13 which shows that after C is dead A has a value[9] which approximates zero, hence in Fig. 14 we make the arrows equal. As Y is in contact with the more "effective" solution we expect it to change more rapidly and we picture the process (after the counter movement) as shown in Fig. 15 *b* from which we plot $y - x$, obtaining

[23] This presumably remains unaffected by the external solution, at least in short experiments.

the curve in Fig. 15 *a* which is a tracing of the latter part of the curve in Fig. 13 (the crossing of the curves at the right in Fig. 15 *b* is apparently a frequent phenomenon at all concentrations and results in the observed curve changing sign for a while in the latter part of its course).

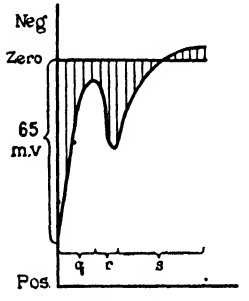

FIG. 11 *a*.

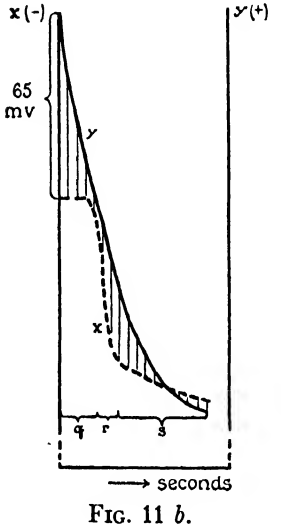

FIG. 11 *b*.

FIG. 11 *a*. Tracing of the latter part of the curve shown in Fig. 9 (after the counter movement). Certain ordinates are drawn for comparison with Fig. 11 *b*.

FIG. 11 *b*. Theoretical interpretation of Fig. 11 *a*. The ordinates as drawn represent the observed potential difference (which is equal to that of Fig. 11 *a*). Fig. 11 *a* shows only the observed potential difference but Fig. 11 *b* shows that this is equal to the difference between the value of x (with negative sign) and that of y (with positive sign). Each ordinate has the same length as the one directly above it in Fig. 11 *a*. The curve begins at the end of the counter movement when the value of A is 65 millivolts. During the period marked *q* the value of x remains stationary while that of y falls off as the curve in Fig. 11 *a* becomes more negative. During the period marked *r* the value of x falls off as the curve in Fig. 11 *a* becomes more positive. During the period marked *s* the curves approach each other, as the curve in Fig. 11 *a* approaches zero; after they cross the curve in Fig. 11 *a* becomes negative.

The behavior in 0.01 M KCl raises an interesting question. In the case of 0.001 M KCl it might be suggested that the layer which changes most rapidly is the one with the greatest potential difference across it. But in the case of 0.01 M KCl where the potential differences across X and Y are equal it would appear that the determining cause is the "effectiveness" of the solution rather than magnitude of the potential difference.

It will be noted that in Fig. 2 (and in Fig. 9 after the counter movement) the first result of the application of chloroform to A is a loss of potential difference. But with 0.01 M KCl even when the value of A before injury is negative we find (disregarding the counter movement) an increase on applying chloroform. For example in the latter part

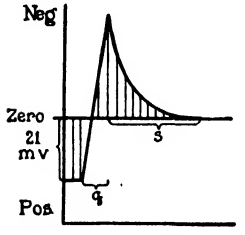

FIG. 12 *a*.

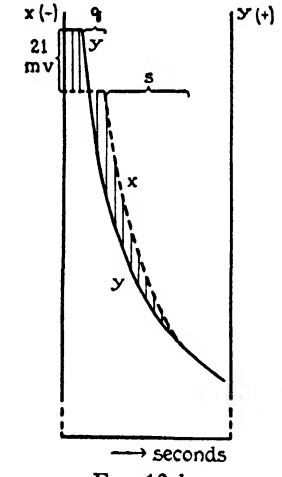

FIG. 12 *b*.

FIG. 12 *a*. Like Fig. 11 *a* but showing a different result.

Fig. 12 *b*. Theoretical interpretation of Fig. 12 *a*. The ordinates as drawn represent the observed potential difference which is equal to that of Fig. 12 *a*. Fig. 12 *a* shows only the observed potential difference but Fig. 12 *b* indicates that this is equal to the difference between the value of x (which has a negative sign) and that of y (with positive sign). Each ordinate has the same length as the one directly above it in Fig. 12 *a*.

During the period marked q the value of x remains constant while that of y falls as the curve in Fig. 12 *a* becomes more negative. During the period marked s the value of x falls off and the two curves approach each other as the curve in Fig. 12 *a* approaches zero.

of the curve in Fig. 16 (when C is dead but A is still normal) A is negative[24] and when chloroform is applied to A[9] it becomes more negative and then commences to fall toward zero. It would seem

[24] The fact that the potential difference across the protoplasm in contact with 0.01 M KCl is sometimes negative (Fig. 16) and sometimes zero (Fig. 13) may be due to variations in sap. It might also be due to variations in X and Y. It has been shown in a former paper[3] that these are not alike, and this accords with the diagram in Fig. 14 for if X and Y were alike we could not make the arrows in this diagram of equal length since sap is approximately equivalent to 0.05 M KCl.

that this would afford a crucial test of the ability of our hypothesis to explain all the facts. As a matter of fact it is precisely what the

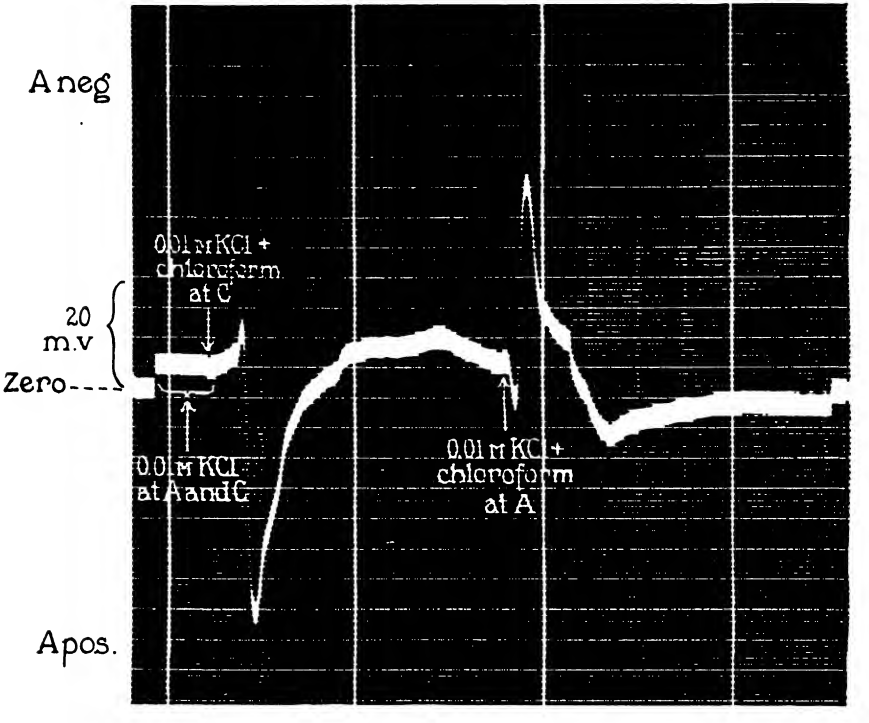

FIG. 13. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at A and C: 0.01 M KCl saturated with chloroform is applied to C and then to A. Just before applying chloroform to A it is slightly negative. When chloroform is applied we see that after the counter movement A becomes more negative, then more positive, and finally approaches zero (C being dead remains constant).

The first part of the curve (resulting from the application of chloroform to C) is similar but with signs reversed because the changes occur at C. After the death of C the curve reaches a fixed value which is practically all due to the living protoplasm at A. Chloroform is then applied to A, giving the death curve already described.

The vertical marks represent 20 second intervals. Selected as typical from over 100 experiments.

hypothesis would lead us to expect, since it demands that in all cases where Y changes more rapidly the first large jump (after the counter

movement) will be in the negative direction even though (as here and in Fig. 8) the value across the protoplasm is already negative.

To interpret Fig. 16 we diagram the protoplasm as in Fig. 3 *b* and

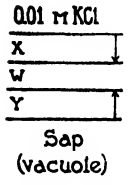

FIG. 14.

FIG. 14. Hypothetical diagram of the condition of the protoplasm in contact with 0.01 M KCl. The potential difference across the protoplasm is zero since the two arrows are equal.

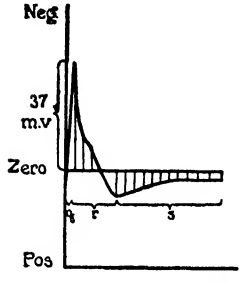

FIG. 15 *a*.

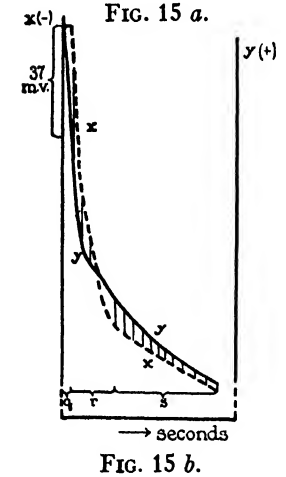

FIG. 15 *b*.

FIG. 15 *a*. Tracing of the latter part of the curve in Fig. 13 (after the counter movement) with certain ordinates drawn for comparison with Fig. 15 *b*).

FIG. 15 *b*. Theoretical interpretation of Fig. 15 *a*. The ordinates as drawn indicate that the observed potential difference is equal to the difference between x (with negative sign) and y (with positive sign) and corresponds to the observed potential difference in Fig. 15 *a* (each ordinate as drawn has the same length as the one directly above it in Fig. 15 *a*). During the period marked q the value of x remains stationary while that of y falls off as the curve in Fig. 15 *a* becomes more negative; during the period marked r the value of x falls off as the curve in Fig. 15 *a* becomes more positive; during the period marked s the two curves approach each other as the curve in Fig. 15 *a* approaches zero (this zero has no relation to the absolute zero of Fig. 15 *b*).

we picture the process (after the counter movement) as shown in Fig. 17 *a* and *b*: *Y* changes more rapidly since it is in contact with the more effective solution.

Let us now consider the effects of a more concentrated solution, namely 0.1 M KCl, as shown in Fig. 18. After *C* is dead we find that

A is negative.[22] We therefore diagram the protoplasm as in Fig. 3 b.[10] Since X is in contact with the more effective solution we expect it to change more rapidly and we therefore picture the process as in Fig. 19 b where x and y fall off in such fashion that when the values of $y - x$

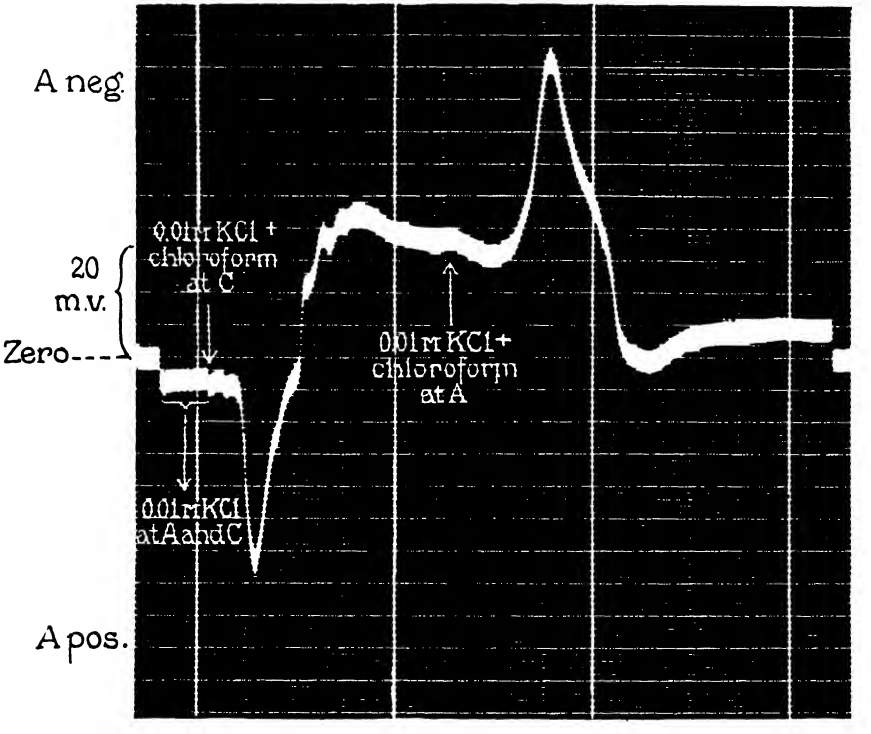

FIG. 16. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at A and C: 0.01 M KCl saturated with chloroform is applied to C and then to A. Just before applying chloroform to A its value is negative: when chloroform is applied we see that after the counter movement it becomes more negative and the potential difference then falls to zero and afterward becomes slightly negative.

The vertical lines represent 20 second intervals. Selected as typical from over 100 experiments.

are plotted we obtain the curve shown in Fig. 19 a, which is a tracing of the latter part of the curve[9] in Fig. 18.

Here also we expect variations similar to those found in other concentrations and this expectation is fulfilled.

The hypothesis would also lead us to predict that 0.005 M KCl would produce effects similar to those of 0.001 M KCl. The experiments show that this is so.

The success of the hypothesis in predicting the behavior of the cell under so many conditions indicates that it may be a useful guide in future investigations.

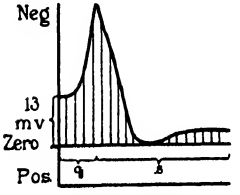

FIG. 17. *a*.

FIG. 17 *a*. Tracing of the latter part of the curve in Fig. 16 (after the counter movement) with certain ordinates drawn for comparison with Fig. 17 *b*.

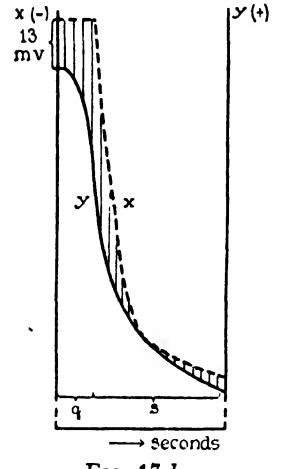

FIG. 17 *b*.

FIG. 17 *b*. Theoretical interpretation of Fig. 17 *a*. The ordinates as drawn indicate that the observed potential difference in Fig. 17 *a* is equal to the difference between x with negative sign and y with positive sign (each ordinate has the same length as the one directly above it in Fig. 17 *a*). During the period marked q the value of x remains stationary while that of y falls as the curve in Fig. 17 *a* becomes more negative. During the period marked s the two curves approach each other as the curve in Fig. 17 *a* approaches zero (this zero has no relation to the absolute zero of Fig. 17 *b*). After this the curves diverge as the curve in Fig. 17 *a* becomes more negative.

It is evident that the hypothesis would be equally satisfactory if in Figs. 3 *a* and *b*, 10 and 14 we should reverse the direction of the arrows and assume that the layer in contact with the more dilute solution is the one which changes more rapidly. It is difficult to test this experimentally with chloroform since it penetrates so quickly as to reach Y almost as soon as X but if we employ a substance which enters less rapidly, first attacking X and slowly making its way through X and W to attack Y, we may be able to decide the question.

For this purpose 0.16 M formaldehyde in 0.01 M KCl was applied at *A* (with 0.01 M KCl at *C*). The result is shown in Fig. 20; it is evident

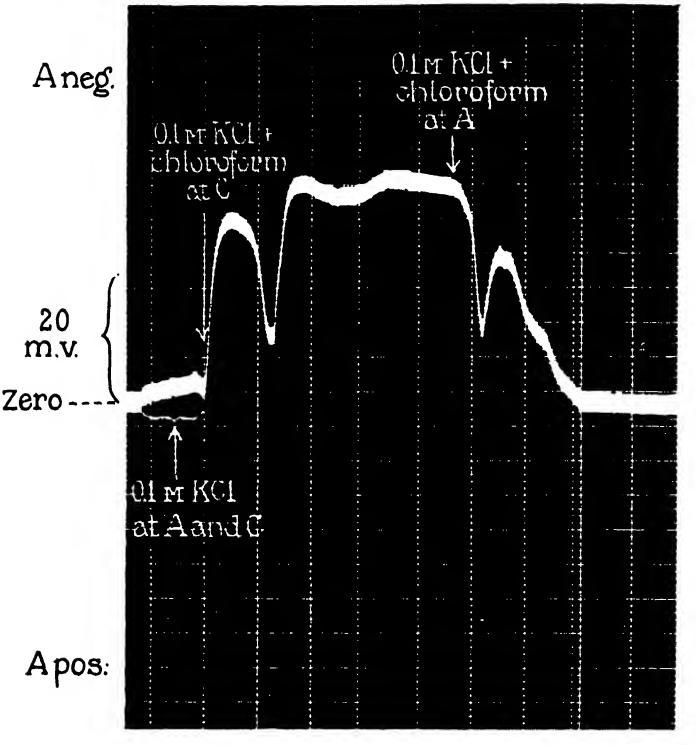

FIG. 18. Photographic record showing potential differences. The experiment starts with 0.1 M KCl at *A* and *C*: 0.1 M KCl saturated with chloroform is applied at *C* and then at *A*. Under the influence of chloroform *A* becomes more positive, then more negative, and the potential difference then falls to zero.

The first part of the curve (resulting from the application of chloroform to *C*) is similar but with signs reversed because the changes occur at *C*. After the death of *C* the curve reaches a fixed value which is practically all due to the living protoplasm at *A*. Chloroform is then applied at *A* giving the death curve already described.

The vertical lines represent 5 second intervals. Selected as typical from over 100 experiments.

that the protoplasm becomes more positive. The action is gradual, presumably because the formaldehyde penetrates slowly. This is just the opposite from the effect of chloroform, which produces a

rapid negative change as shown in Fig. 21 where 0.01 M KCl saturated with chloroform was applied at C causing its value to become more negative (it appears on the record to become more positive because the curve records the state of A with reference to C, hence the signs are reversed): 0.16 M formaldehyde was then applied at A which slowly became more positive.

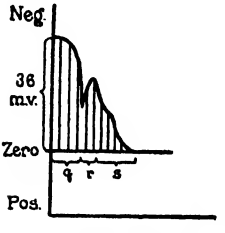

FIG. 19 *a*.

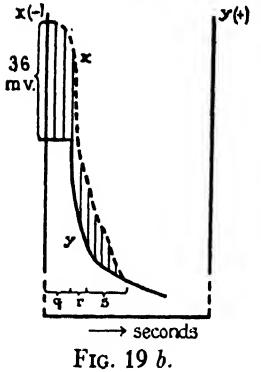

FIG. 19 *b*.

FIG. 19 *a*. Tracing of the latter part of the curve in Fig. 18 with certain ordinates drawn for comparison with Fig. 19 *b*.

FIG. 19 *b*. Theoretical interpretation of Fig. 19 *a*. The ordinates as drawn indicate that the observed potential differences in Fig. 19 *a* are equal to the difference between x with negative sign and y with positive sign (each ordinate has the same length as the one directly above it in Fig. 19 *a*). During the period marked q the value of y remains constant while that of x falls off as the curve in Fig. 19 *a* becomes more positive; during the period marked r the value of y falls off as the curve in Fig. 19 *a* becomes more negative; during the period marked s the two curves approach each other as the curve in Fig. 19 *a* approaches zero (this zero has no relation to the zero of Fig. 19 *b*).

These results indicate that the X arrow has the direction shown in the figures. This does not seem to be in harmony with the widely accepted view that the inside of the plasma membrane is negative to the outside[25] for in that case the arrow at X would have to be reversed. The idea that the inside of the plasma membrane is negative was adopted to account for the negative current of injury but it seems probable that in *Nitella* at least the negative current of injury described in the literature can be accounted for in a different way. The

[25] If, as we assume, the inside of X is positive we should find on inserting a capillary through X so as to lead off from the inside of X to the outside that the positive current would flow from the inside through the capillary and the electrometer to the outer surface of X.

negative currents of injury described in this paper may be too brief to correspond to the traditional negative current of injury referred to in the literature and it is questionable to what extent they could be observed in cells of ordinary size with the methods usually employed.

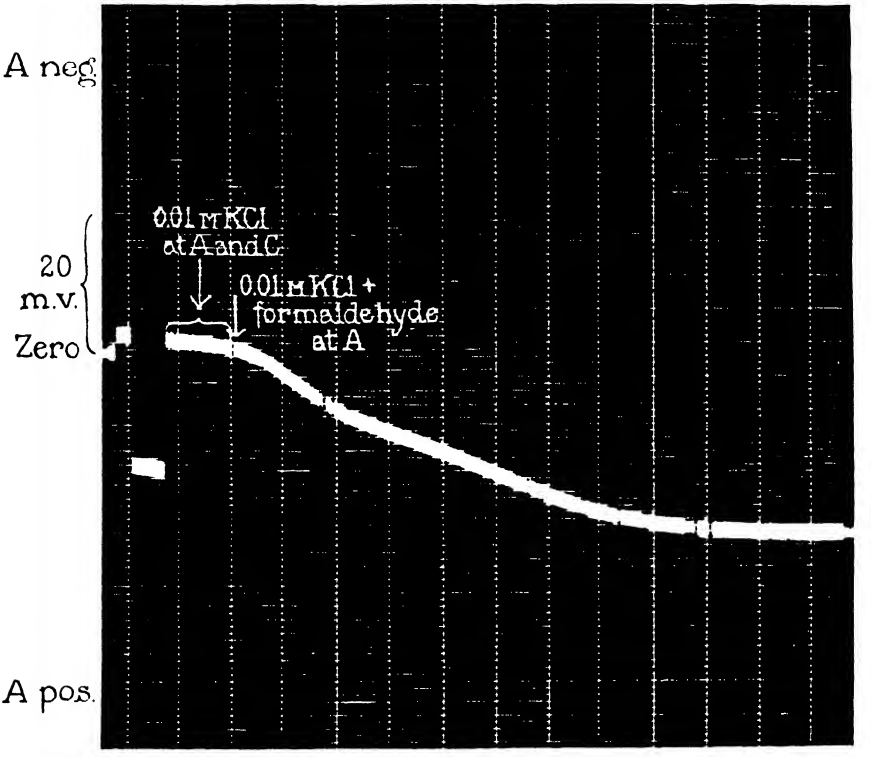

FIG. 20. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at *A* and *C*: then 0.16 M formaldehyde in 0.01 M KCl is applied at *A* (with 0.01 M KCl at *C*). Formaldehyde makes *A* more positive but the action is gradual.

The vertical lines represent 5 second intervals. Selected as typical from 30 experiments.

In order to obtain results comparable with those described in the literature it would seem to be necessary to make experiments with portions of a plant of *Nitella* consisting of two or more cells. If we do this we find, on cutting or crushing a cell at one end, that sap, escaping at the opposite end and coming in contact with a neighboring intact cell, causes the injured cell to appear more negative and this

condition may persist for a long time. This will be fully discussed in a subsequent paper.

It will be remarked that in the previous discussion no account is taken of any possible potential differences in W: this does not imply that none exist but only that they are regarded as negligible for our present purpose. It would be possible to set up another hypothesis which would attribute more importance to W. For example, in the

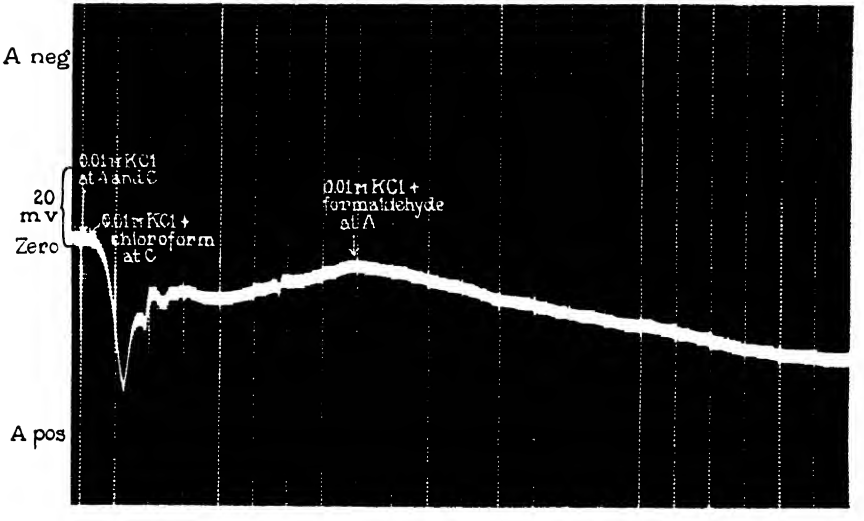

FIG. 21. Photographic record showing potential differences. The experiment starts with 0.01 M KCl at A and C. The application of 0.01 M KCl saturated with chloroform to C causes it to become more negative (the curve becomes positive because the signs are reversed owing to the fact that the change occurs at C but the curve shows the state of A rather than that of C). During this time A is in contact with 0.01 M KCl. Formaldehyde (0.16 M in 0.01 M KCl) applied to A causes it to become more positive. Compare with Fig. 20.

The vertical lines represent 5 second intervals. Selected as typical from 10 experiments.

case of 0.001 M KCl we might suppose that both X and Y are simultaneously destroyed leaving W exposed to 0.001 M KCl on the outside and to sap on the inside: if W reacts in the opposite way[26] from

[26] Beutner (Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920), experimenting on organic substances immiscible with water, found that basic substances give an effect opposite to that of acid substances with such salts as KCl.

X and *Y* (*e.g.*, if, in contact with *W* 0.001 M KCl is negative to sap and to 0.05 M KCl) the value across the protoplasm would change from positive (as in Fig. 4 *a*) to negative (as in Fig. 4 *b*).

But this hypothesis would not account for such cases as those shown in Figs. 9 and 18 where the curve moves first in one direction and then in the other, nor for Fig. 16 where the value of *A* is negative and becomes more so, nor would it explain why a spot treated with sap + chloroform commonly behaves as shown in Fig. 8, since in that case *W* would have sap on both sides and unless we assume that *W* is composed of two unlike layers we should expect that when chloroform is applied the potential difference would at once fall to zero.

Another possible explanation is that the application of chloroform alters the protoplasm in such fashion as to let sap pass out (at least momentarily) and come in contact with the outside of *X* without altering its power to give a potential difference. If we had 0.001 M KCl at the outside at the start it would be changed to the equivalent of something between 0.001 M and 0.05 M KCl by the coming out of sap. This would lessen the potential difference and might even make it negative. On the other hand 0.1 M KCl which gives a negative value across the protoplasm would be diluted by the coming out of sap and would become less negative but it could never become temporarily positive, as often happens in cutting, because if the 0.1 M KCl were completely changed to sap the value would still be negative (as shown in a previous paper[3]). In addition this hypothesis fails to explain why a spot treated with sap saturated with chloroform commonly behaves as shown in Fig. 8.

After the completion of this paper we received through the kindness of Professor Jost a reprint of his paper[27] which deals with *Chara* and *Valonia*. He makes no mention of experiments with chloroform but his results with ether, ethyl alcohol, amyl alcohol, cutting and crushing agree (except in minor details) with ours which have been repeated many times during the last 5 years. We think it desirable, however, to make it clear that we interpret the lasting negative current of

[27] Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch., Abteilung B*, 1927, Abhandlung 13, Nov.

injury, observed when we lead off with dilute solutions from a cut or crushed cell, as due to an effect upon the neighboring intact cell (from which we are in effect leading off at one point with a dilute solution and at another with sap escaping from the injured cell, the circuit passing through the injured cell). If the solution applied to the uninjured cell is as "effective" as sap the current of injury will be of relatively brief duration. But if the solution applied to the uninjured cell is more "effective" than sap the current of injury may last for a long time and will be positive.

In view of the fact that the current of injury is sometimes explained by differences in acidity it may be well to state that varying the pH value from 5 to 8 produces relatively little effect as long as the concentration of cations other than H^+ is not changed.

SUMMARY.

Experiments on single multinucleate cells of *Nitella* show that the current of injury may be made positive or negative. For example, with chloroform in 0.1 M KCl the current of injury is positive but with 0.001 M KCl it is negative.

The changes which occur during the process of death receive a simple explanation upon the basis of the theory of protoplasmic layers. It seems possible that each layer has a death curve of simple and regular form, the more rapid alteration of the outer layer producing a positive current of injury and the more rapid alteration of the inner giving rise to a negative current of injury.

[From THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, March 24, 1928, Vol. xc, No. 12, pp. 906–908.]

BACTERIAL ALLERGY (HYPERERGY) TO NONHEMOLYTIC STREPTOCOCCI IN ITS RELATION TO RHEUMATIC FEVER.*

BY HOMER F. SWIFT, M.D.; C. L. DERICK, M.D. AND C. H. HITCHCOCK, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The subject of bacterial allergy in its relation to certain phenomena of infectious diseases has assumed increasing importance in recent years. In our studies of rheumatic fever the view that such an allergy has an important bearing in the pathogenesis of the disease has become more and more prominent. Several years ago one of us[1] called attention to the similarity between many of the manifestations of rheumatic fever and those of syphilis and tuberculosis. The anatomic features of these last two diseases have been thoroughly established as allergic in their pathogenesis. If, therefore, one had a conception of rheumatic fever as an analogous disease, it seemed only logical to determine whether the micro-organisms suspected of playing an etiologic rôle could be brought into relationship with any allergic state. In this connection we[2] have been able to show that by suitable inoculation with nonhemolytic streptococci it is possible to induce in rabbits a hyperergic condition comparable to that obtained in animals inoculated with tubercle bacilli. When the hyperergic rabbits are subsequently inoculated intracutaneously with amounts of streptococci too small to cause grossly appreciable lesions in normal animals, they react with papules from 10 to 15 mm. in diameter and from 1 to 3 mm. thick. If their anesthetized corneas

* Read in a symposium on rheumatic fever before the Philadelphia Pathological Society, Dec. 8, 1927.

1. Swift, H. F.: Rheumatic Fever, Am. J. M. Sc. **170**: 631 (Nov.) 1925.

2. Andrewes, C. H., Derick, C. L., and Swift, H. F.: The Skin Response of Rabbits to Nonhemolytic Streptococci: I. Description of a Secondary Reaction Occurring Locally After Intradermal Inoculation. J. Exper. Med. **44**: 35 (Jan.) 1926. Derick, C. L., and Swift, H. F.: Hyperergic Tissue Response to Nonhemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. **25**: 222, 1927.

are lightly scarified and a drop of culture is instilled into the conjunctival sac, an interstitial keratitis is produced which varies in intensity and persists from three to fifteen days according to the degree of hypersensitiveness of the animal. Similar inoculations in normal rabbits have not produced a comparable keratitis. This hyperergic state can be maintained fairly well in animals by the production of infected agar foci. One of the most interesting features of this condition is that it is not specific when considered from the standpoint of strain specificity; that is, a strain A which has certain cultural and immunologic characteristics may induce a state in an animal in which it is possible to demonstrate hypersensitiveness to strains B, C or D, which are within certain limits widely separated from the original allergizing strain. These animals also show skin hypersensitiveness to filtrates, not only of the organisms with which they have been rendered hyperergic but also to those of the more distantly related strains. This immediately suggested that the variability in the cultural and immunologic characteristics of various types of nonhemolytic streptococci and even of hemolytic streptococci which have been isolated from patients with rheumatic fever was of less significance than one might *a priori* think probable. In 1917, Swift and Kinsella[3] showed that there was a decided heterogeneity in the strains they recovered; and since that time these observations have been confirmed repeatedly by us. On more than one occasion we have been able to recover two or more immunologically different strains of nonhemolytic streptococci, either from the blood or subcutaneous nodules of patients during life, or from material obtained post mortem. Birkhaug[4] has recently brought forth evidence to show that patients who are "stigmatized by rheumatic fever" show skin hypersensitiveness to filtrates of a special group of anhemolytic streptococci. We have been able to confirm these observations, but in addition have found that most patients in the acute stages of the disease, and many who have apparently re-

3. Swift, H. F., and Kinsella, R. A.: Bacteriologic Studies in Acute Rheumatic Fever, Arch. Int. Med. **19**: 381 (March) 1917.

4. Birkhaug, K. E.: Rheumatic Fever: Bacteriologic Studies of a Non-Methemoglobin-Forming Streptococcus with Special Reference to Its Soluble Toxin Production, J. Infect. Dis. **40**: 549 (May) 1927.

covered, also show a similar skin hypersensitiveness to filtrates of certain strains of *Streptococcus viridans*. Zinsser and Grinnell[5] assert that in autolysates of various bacteria are set free the substances which stimulate the allergic state, and that such autolysates are the best reagents for testing hypersensitiveness. We have found that the strains described by Birkhaug or similar strains die more quickly in broth cultures and autolyze more readily than do most other nonhemolytic streptococci. In this fact may rest a possible explanation of the greater ease of obtaining a skin reactive substance from these inulin-fermenting, anhemolytic strains.

Another interesting feature of the reacting substance is that in many instances this so-called toxin is not inactivated by boiling one hour; indeed, in the majority of patients with active rheumatic fever the skin reaction with this boiled filtrate has been more marked than with control unboiled filtrate in the same dilution. We have also found that the rabbits made hyperergic in the manner described show similar phenomena when tested with these filtrates in their various forms.

Rabbits which are highly hyperergic to nonhemolytic streptococci show reactions, following intravenous injection of these streptococci, similar to those seen in tuberculous animals injected intravenously with tubercle bacilli or tuberculin. This suggested to us the possibility of testing the reactions of patients to the intravenous injection of very small doses of killed streptococci or of their products, and we[6] have found that these reactions generally resemble those of tuberculous patients or animals following injections of small doses of tuberculin. The febrile reaction usually develops slowly and reaches its maximum within ten to twenty-four hours following the injection. This is in distinct contrast to the nonspecific protein shock reactions observed after intravenous injection of typhoid bacilli. The reac-

5. Zinsser, Hans, and Grinnell, F. B.: The Antigen Involved in Pneumococcus Allergy, J. Bact. **14**: 301, 1927.

6. Swift, H. F., Hitchcock, C. H., and Derick, C. L.: General Tuberculin-Like Reactions in Rheumatic Fever Patients Following Intravenous Injection of Streptococcus Vaccines or Nucleoproteins, Proc. Soc. Exper. Biol. & Med. **25**: 312, 1928.

tions observed by Small[7] in patients with rheumatic fever following subcutaneous injection of vaccines prepared with *Streptococcus cardioarthritidis* closely resemble tuberculin reactions. Many physicians have reported the occurrence of nonspecific protein shock reactions in rheumatic patients following the injection of a number of substances, but so far as we can determine these reactions occur after a shorter interval and run a shorter course than do the tuberculin-like reactions just mentioned. We feel, therefore, that these reactions furnish additional evidence in support of the hypothesis that there is in the patient with rheumatic fever a state comparable to that of the rabbit made hyperergic to certain nonhemolytic streptococci.

We have also determined that the hyperergic state in rabbits is induced most readily by the production of focal lesions, and is maintained for months by making an agar focus infected with the allergizing streptococci. Intravenous inoculation,[8] on the other hand, fails to induce this hyperergic state. The presence of a focus of infection therefore, is a most important factor in inciting this state. Here, again, there is a striking parallelism between the condition of these rabbits and that of patients with rheumatic fever. There is much clinical evidence to support the contention that focal infection has an important bearing on the evolution of this disease, but up to the present the importance of the focus was thought to rest in its rôle as a nidus from which the virus was disseminated throughout the body. Our conception of the focus, on the other hand, is of an area where the allergizing substance is produced and whence it is spread to sensitize the various tissues. This conception does not deny that virus may also gain entrance to the blood stream from the focus, but stresses the allergizing effect of such a focus. From this hypothesis it is easy to understand that the state of the tissues of the patient is the most characteristic feature of the disease and that the specificity of the streptococci recovered may play a relatively unimportant rôle.

One of the causes of confusion resulting from the reports of the results of different observers has been the variety of streptococci

7. Small, J. C.: Personal communication to the authors and at the meeting of the Philadelphia Pathological Society, Dec. 8, 1927.

8. Swift, H. F., and Derick, C. L.: Immune Tissue Response to Nonhemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. 45: 224, 1927.

which have been isolated from patients with rheumatic fever. Each worker is naturally inclined to attribute a specific etiologic rôle to the strain isolated by him. The hypothesis that in rheumatic fever there is a hypersensitive state, which is not strictly strain specific, but which embraces a wide range of streptococci, reconciles these divergent observations. It makes understandable the possibility that the micro-organisms, described by the following workers, to mention only a few, may indeed have been the exciting agents of the active disease in the patients from which they were recovered: the *Streptococcus cardioarthritidis* of Small;[9] the inulin-fermenting anhemolytic streptococcus of Birkhaug;[4] the strains described by Clawson;[10] the *Micrococcus rheumaticus* of Poynton and Paine,[11] and the three types found by Rosenow.[12] It also explains the possible etiologic rôle of the strains recovered by us in the past fifteen years, despite their great cultural and immunologic variation. The existence of such a hyperergic state, as suggested by the observations of Mackenzie and Hanger,[13] in persons who are not suffering from rheumatic fever, may also explain the frequent finding of pathologic evidence of the disease in subjects who have failed to show the classic symptoms of the disease during life.[14] We feel, therefore, that this hypothesis has now enough support to entitle it to consideration as a theory. It is interesting in reviewing Menzer's[15] work to note that he had a somewhat similar conception of the disease at a time before allergy or the

9. Small, J. C.: The Bacterium Causing Rheumatic Fever and a Preliminary Account of the Therapeutic Action of Its Specific Antiserum, Am. J. M. Sc. **173:** 101 (Jan.) 1927.

10. Clawson, B. J.: Studies on the Etiology of Acute Rheumatic Fever, J. Infect. Dis. **36:** 444 (May) 1925.

11. Poynton, F. J., and Paine, A.: The Etiology of Rheumatic Fever, Lancet **2:** 861, 1900.

12. Rosenow, E. C.: The Etiology of Acute Rheumatism, Articular and Muscular, J. Infect. Dis. **14:** 61, 1914.

13. Mackenzie, G. M., and Hanger, F. M.: Allergic Reactions to Streptococcus Antigens, J. Immunol. **13:** 41 (Jan.) 1927.

14. Von Glahn, W. C.: Rheumatic Disease of the Heart, Arch. Path. & Lab. Med. **3:** 355 (Feb.) 1927.

15. Menzer, A.: Serumbehandlung bei acutem und chronischem Gelenkrheumatismus, Ztschr. f. klin. Med. **47:** 109, 1902.

allergic state was recognized. He thought that the general dissemination of the streptococci occurred at the time of tonsillitis or other initial lesion which often precedes an attack of rheumatic fever, and that only at the time that the patient developed immune bodies did the general metastatic manifestations of the disease appear. Weintraud[16] also considered hypersensitiveness as being of importance in the pathogenesis of the disease, but he argued from the point of view of serum disease rather than from that of bacterial allergy.

While it is true that serum disease and rheumatic fever possess in common the condition arthritis, this does not necessarily indicate that the two diseases have a similar pathogenesis. The points of dissimilarity between the arthritis of the two diseases are many, and may be explainable on the basis of the differences between protein hypersensitiveness and bacterial allergy. Dochez and Stevens[17] have recently established in animals infected with *Streptococcus erysipelatis* the existence of two types or phases of hypersensitiveness, one to a toxin which can be neutralized in vitro, and the second to some other bacterial product which they were unable to neutralize. Birkhaug[4] stated that he was able to neutralize by means of antitoxic serum the toxic filtrate he obtained; but so far we have been unable to demonstrate a comparable neutralization in patients who were suffering from active rheumatic fever. There may be in patients with rheumatic fever two phases of hypersensitiveness to nonhemolytic streptococci; if so, our observations indicate that during the active stages of the disease the allergy is predominantly of the second type. Whether or not these patients will pass into a condition later in which the skin allergic reactions can be prevented by specific neutralization of the toxic filtrate with an antitoxic serum in vitro is a question that can be answered only after continued observations.

16. Weintraud, W.: Ueber die Pathogenese des akuten Gelenkrheumatismus, Berl. klin. Wchnschr. **50**: 1381, 1913.

17. Dochez, A. R., and Stevens, F. A.: Studies on the Biology of Streptococcus: VII. Allergic Reactions with Strains from Erysipelas, J. Exper. Med. **46**: 487 (Sept.) 1927.

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THE EFFECT OF ANTI-RHEUMATIC DRUGS ON THE ARTHRITIS AND IMMUNE BODY PRODUCTION IN SERUM DISEASE.

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Serum sickness, the group of symptoms which follows the administration of foreign serum to human beings, was first extensively observed and recorded clinically by Von Pirquet and Schick (1) and later by other groups of workers. These studies concerned themselves entirely with the clinical and serological aspects of the disease; but up to the present little or no attempt has been made to obtain a method of treatment to prevent or lessen the severity of this illness. The present study was undertaken, therefore, with this in view. This work was suggested by the findings of Boots and Swift (2), who showed that in patients with serum sickness the involved joints contained a cellular exudate in which the proteins of the horse serum could be demonstrated. They also pointed out that salicylates had little effect upon the course of the illness when administered after the onset of arthritis.

During the past two years we have studied the serum-treated patients in this Hospital to determine, first the influence of the early institution of anti-rheumatic therapy upon the course of the disease, and, second the effect of such therapy upon the antigen and antibody content of their sera.

Neocinchophen, because of its anti-exudative influence in acute rheumatic fever (3) and the ease with which it is tolerated by patients, was employed in the first twenty-five cases; it was replaced by aspirin in the last nine. Depending upon the age and weight of the patient and his tolerance for the agent, neocinchophen was used in amounts of 8 to 10 grams daily, aspirin in doses of 5 to 6 grams. Drug treatment was usually begun from 24 to 48 hours after the last serum injection, and continued for 10 to 14 days. As the advantages of

early and prolonged therapeusis quickly became evident, this schedule was strictly followed with the exception of four instances in which either dosage or duration was insufficient. As identical results were obtained with both drugs, the two series have been analyzed as one group. All patients were carefully examined daily for the appearance of lymphadenopathy, urticaria and arthritis, and the signs and symptoms, whether positive or negative, were charted along with the temperature and pulse records. The degree of intensity of the respective manifestations was recorded by using the − sign, or one or more + signs, according to their severity.

As different observers have reported variations in the frequency of the individual manifestations of serum disease, we have thought it best to use as controls for this series the incidence of the various symptoms in the patients treated in this Hospital previous to the time of this study. In this way the elements of dosage of serum and type of infection—lobar pneumonia—for which it was given were constant. The records of 65 such controls were analyzed and compared with those of 34 patients subjected to anti-rheumatic therapy. In 30 of the latter, treatment was continued for a sufficient period; in 3 others its premature cessation was in each instance quickly followed by the development of arthritis. One of these is represented as the case of severe arthritis in the column marked "Treated" of table 1; in one there was moderate and in another a slight arthralgia. In a fourth patient, moderate arthritis developed because of insufficient, even though adequately prolonged, treatment.

Table 1 shows the frequency and severity of arthritis in these two groups. Of the treated patients, over 82 per cent showed little or no arthritis as compared with only 50 per cent in the untreated group. The percentages with moderately severe arthritis were about the same in each series, i.e., 14.6 per cent of the treated patients and 15.2 per cent of the untreated. Further, it will be noted that only the one patient mentioned above, who was insufficiently treated, or 3 per cent of the treated patients, had a severe arthritis, compared with over 30 per cent among those untreated. Thus, as is well shown in this table, the lessening in the frequency and severity of the arthritis in the treated patients is quite evident. No comparison as to the duration of joint involvement in the two series has been attempted.

In table 2 is given the comparison of the degree and frequency of urticaria in the same two groups. Of the untreated patients, 7.7 per cent showed little or no urticaria as compared with 11.8 per cent of those treated; 31.9 per cent of the untreated patients showed a mild to moderate urticaria as compared with 14.7 per cent of the treated;

TABLE 1.

Comparison of Arthritis in Treated and Untreated Patients.

Untreated patients			Treated patients	
Degree of severity	Number	Per cent of total	Number	Per cent of total
−	27	40.9	22	64.7
±	6	9.0	6	17.7
+	5	7.6	2	5.9
++	5	7.6	3	8.7
+++	12	18.2	0	0
++++	8	12.1	1	3.0
No mention	3	4.5		
Totals.....	66	100 per cent	34	100 per cent

TABLE 2.

Comparison of Frequency and Severity of Urticaria in Treated and Untreated Patients.

Untreated patients			Treated patients	
Degree of severity	Number	Per cent of total	Number	Per cent of total
−	2	3.0	4	11.8
±	3	4.7	0	0
+	11	16.7	2	5.9
++	10	15.2	3	8.8
+++	24	36.4	10	29.4
++++	16	24.0	15	44.1
Totals.....	66	100 per cent	34	100 per cent

while 60.4 per cent of the first series showed severe urticaria as compared with 73.5 per cent of the second.

Although there was little difference in the frequency of urticaria in the two series, this symptom was more intense among the treated patients. As shown in the table, the number of patients with severe urticaria was 13 per cent less among those not receiving anti-rheumatic

drugs. Whether these drugs, which are known occasionally to cause exudative dermatoses in susceptible individuals, may exert a synergic influence and thus increase the severity of the exudation into the skin of patients with serum disease, is a question we cannot answer with certainty. Such an explanation is, however, not unreasonable. But in spite of this statistical evidence, the great comfort resulting from the nearly complete elimination of arthritis more than counterbalances this undesirable effect.

Adenopathy was present with sufficient frequency to demonstrate that the therapy was apparently without effect upon its incidence. As only rarely is it a source of discomfort to the patient, it will not be further considered. Febrile reactions of varying intensity were quite regularly observed. How far they were influenced by the well-known antipyretic effect of the drugs employed it would be unprofitable to conjecture at the present time.

As soon as it was evident that a definite anti-arthritic influence of anti-rheumatic drugs could be demonstrated in serum sickness, it became desirable to determine whether there was any parallelism between this phenomenon and the immunological manifestations of the disease.

The sera of twenty treated patients were therefore studied with respect to the elimination of horse serum and the appearance of anti-horse precipitin. For this purpose blood was obtained as soon as anti-rheumatic therapy was instituted, or just prior thereto, and at intervals thereafter of four to seven days during the remainder of the hospitalization. In a number of instances it was possible to procure further specimens at varying intervals following discharge.

Since the early studies of Hamburger and Moro (4) various observers (5) have commented upon the antigen-antibody relationships in serum disease. Longcope and Rackemann (6) observed that in this condition anaphylactin and precipitin for horse serum appeared in the blood stream shortly before recovery, and that the occurrence of antibody in high titer was accompanied by rapid diminution or complete disappearance of the circulating antigen. In the serum of patients who failed to develop serum sickness such antibodies were not found. In their opinion the neutralization or destruction of the antigen by these antibodies was the determining factor in recovery.

More recently Mackenzie and Leake (7), following a careful study of nineteen patients to whom serum had been administered, were able to distinguish three types of serological behavior. In the largest group were included those individuals who suffered from severe serum disease, and in whose sera precipitin appeared. Under these conditions the horse serum was found to disappear from the circulation near the end of the disease, at a time when the precipitin was present in high titer. In the second group were included a few patients who failed to develop serum disease, and in whose sera no precipitin could be demonstrated. In these patients the antigen persisted in the blood stream for extended periods of time. The third group was intermediate: its members, although suffering from serum disease, produced antibodies only in low titer, and antigen could consistently be demonstrated in their sera, though in reduced concentration with the passage of time.

In spite of differing theoretical interpretations, there has been no dispute concerning the actual serological findings of untreated serum disease. It has therefore seemed permissible to utilize the observations of Longcope and Rackemann and of Mackenzie and Leake as controls upon the results reported in this study, especially as the amounts of unconcentrated serum administered were practically the same in the different groups.

METHODS

Anti-sera were prepared by daily subcutaneous injections into rabbits of undiluted horse serum in doses of 0.2 cc. As soon as a precipitin titer of 1:40,000 or better was obtained the animals were exsanguinated, usually one week following the final injection, and the sera were stored in the ice-box without preservative. To avoid confusion from the possible presence of antigen in the blood stream at the time of bleeding, each serum was titrated in ascending dilutions against each of the others. No serum was used with which any suspicion of clouding was observed.

To test for the presence of antigen (horse serum) in the patients' sera, 0.2 cc. of a mixture of equal parts of anti-serum and normal salt solution was placed into each of a series of small tubes, and the human serum to be tested was added in 0.2 cc. amounts in dilutions ranging from 1:2 to 1:200,000. All readings were expressed in terms of the final dilutions of human serum resulting. In view of the small amount of anti-human precipitin present in most high titer rabbit anti-horse sera, many control series were made with sera from healthy subjects.

In addition, as a check against deterioration of the precipitin, normal horse serum was titrated against the anti-serum employed each day that tests were carried out. The same anti-serum was always utilized for testing the entire series of bleedings from any given patient.

The same general technique was followed in testing the sera of patients for the presence of antibody (anti-horse precipitin). Into each of a series of small tubes was placed 0.2 cc. of the undiluted human serum, and normal horse serum was added in 0.2 cc. amounts in dilutions ranging from 1:2 to 1:200,000. Results were expressed in terms of the final dilutions of horse serum. A control series were always employed in which serum from a normal human subject was tested against the same dilutions of horse serum.

All tests were incubated in the water-bath at 37°C. for a period of two hours, following which they were left over night in the ice-box. Readings were made upon the following morning.

Usually the sera were tested within a few days of the bleeding. A few specimens were preserved in the ice-box for periods varying up to five weeks before being titrated. Several comparative observations revealed no significant differences in the results obtained before and after the lapse of such an interval.

RESULTS

With respect to their immunological behavior, our series of treated and serologically tested cases may easily be divided into three groups.

Of these the first corresponds with group 2 of Mackenzie and Leake, and includes those patients, four in number, in whom there was little or no evidence whatever of serum disease. The sera of these individuals failed consistently to reveal the presence of antibody, and the titer of the antigen remained at a high level throughout their stay in the hospital, showing but a slight diminution toward the time of discharge. Two of these four were observed at intervals following their discharge; in one case the antigen had disappeared from the blood stream at the end of three months, while in the other traces were still present at the end of two and a half months. Antibody was not detected at any time. Chart 1 represents a typical member of this group.

The second group, the largest of all, comprises those patients, eleven in number, in whom signs of serum disease were indubitably present, but who through adequate therapy failed to develop arthritis. Chart 2 shows a typical example of this group. Four of them failed to show precipitin during their hospitalization, while in three others

it was present during this period only in evanescent traces. In three more it was found to the extent of 1:40, while in only one instance was a titer of 1:250 reached. In only one of the seven who developed antibody did this appear in detectable amounts prior to the subsidence of the initial urticaria. The antigen (horse serum) titer in the serum remained high throughout the entire period, apparently quite uninfluenced by the development of small amounts of precipitin;

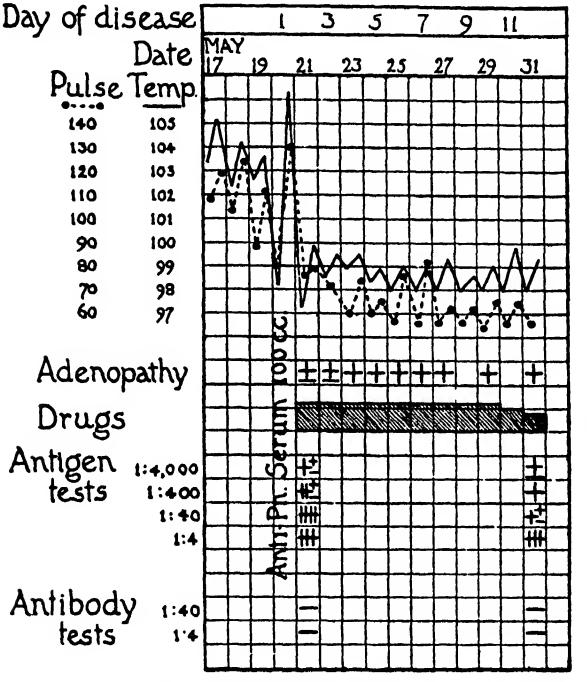

CHART 1. NO SERUM DISEASE; NO ANTIBODY FORMATION

Patient received 6 gm. aspirin per day over period indicated

toward the end of the hospitalization there was some diminution in the figure, but no more than was observed in the cases of the first group. Seven of these patients were observed at intervals for three months following discharge; in five instances there was complete disappearance of antigen by the end of this period, while in the sixth case only traces were found. The seventh patient still harbored demonstrable amounts of horse serum at this time, but at the end of two more

months this had vanished. With a single exception, and that questionable, precipitin was never demonstrated during the follow-up period.

Into the third group may be placed five patients in whom arthritis of varying degrees of severity developed.

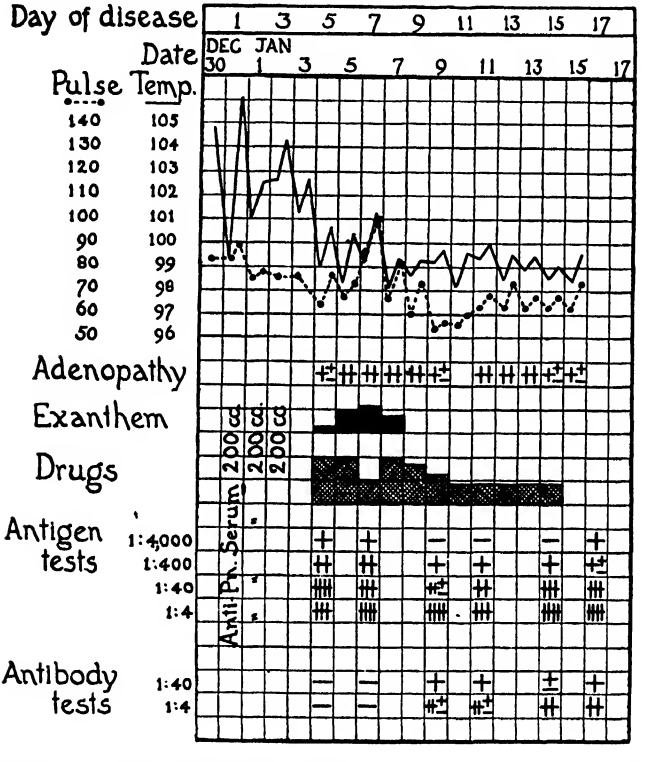

CHART 2. SERUM DISEASE; NO ARTHRITIS; LOW ANTIBODY FORMATION

Patient received neocinchophen maximum 10 gm. per day. Each large block represents 5 gm.

In the first case there was present on three separate days a mild arthralgia characterized principally by stiffness and vague pains, with but little resemblance to the severe arthritis of serum sickness; hence there was a reasonable doubt as to whether the symptoms should be so construed. At no time was antibody present in this patient's serum, and the antigen titer remained high during her hospitalization.

The second patient was one in whom moderately severe arthritis developed on the twenty-fifth day following serum administration.

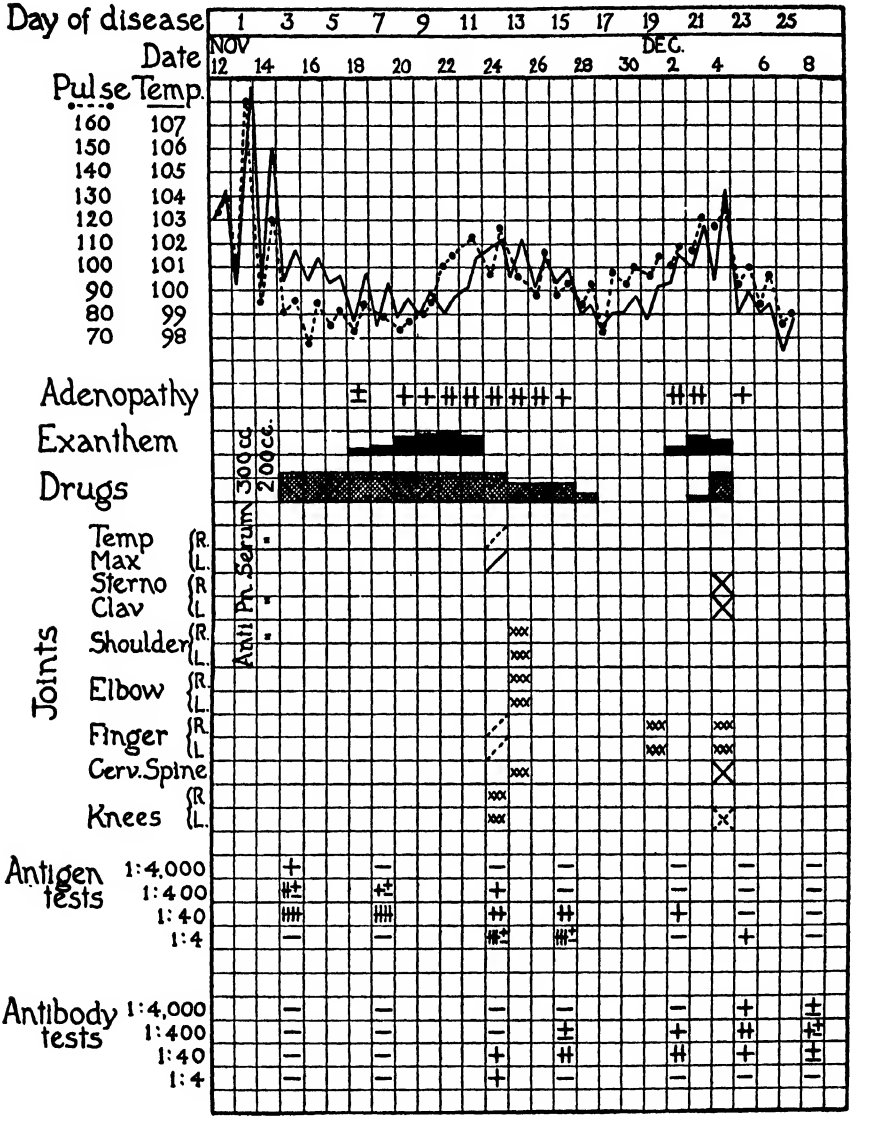

CHART 3. SERUM DISEASE; MILD ARTHRITIS; MODERATE ANTIBODY FORMATION

Patient received neocinchophen 6 gm. per day over period indicated. / represents pain. \ represents tenderness. xxx represents stiffness.

Throughout the preceding period the antigen titer had remained high; immediately following the subsidence of the arthritis, however, a sharp drop was found to have taken place, and three weeks later only traces of horse serum could be demonstrated. At no time was it possible to detect precipitin. This was the only occasion upon which arthritis of any severity appeared in the face of adequate anti-rheumatic therapy.

The other three patients suffered moderately severe arthritis as the result of insufficient treatment. In each case antibody appeared closely upon the subsidence of the urticaria, and at the time of the arthritis had reached a titer of about 1:400. During the period of the arthritis a sharp drop took place in the antigen curve, though it

TABLE 3.

Relationship between Development of Antibodies and Appearance of Arthritis.

	Number of patients	Therapy		Precipitin		
		Adequate	Inadequate	None	1:4 to 1:250	1:400 or higher
No serum disease	4	4		4	0	0
Serum disease without arthritis	11	11		4	7	0
Serum disease with arthritis	5	2		2*	0	0
			3	0	0	3
Totals	20	17	3	10	7	3

* Arthritis very slight in one case.

never reached the base line. Following the arthritis the antibody titer remained at 1:400 and in the case of one patient, shown on chart 3, who suffered a relapse, it reached the figure of 1:4,000. Both antigen and antibody disappeared from the serum of one patient by the end of a month, and from that of a second by the end of two months. With one exception the formation of circulating antibody in titer approximating 1:400 seemed to be the necessary condition for the development of arthritis. The significance of this fact will be discussed below.

In table 3 are presented in condensed form the relationships in these drug treated patients between the existence of arthritis and the development of circulating antibody.

DISCUSSION

The foregoing observations have revealed two rather interesting phenomena. Early, adequate and sufficiently prolonged administration of aspirin or neocinchophen to patients who have received large amounts of anti-pneumococcus horse serum usually results in the prevention of one clinical manifestation of serum disease—the arthritis. Under similar therapeutic conditions there is a failure on the part of the patient to develop circulating antibodies in a concentration comparable with that shown by unmedicated controls. The conclusion seems justified, therefore, that there is some causal relationship between these two facts. It is important that the drugs be started soon after the serum treatment is discontinued; for we have frequently observed the occurrence of severe arthritis when the drugs were not given until later, even though the patients were saturated to the point of toxicity, and also when the medication was insufficiently prolonged. The clinical effect, therefore, is somewhat different from that observed in rheumatic fever, in which a severe arthritis usually disappears shortly after the exhibition of full therapeutic doses.

The observations of Boots and Swift (2) indicate that the so-called arthralgia is a true inflammatory process, or at least is characterized by the presence of cellular exudate in the synovial fluid. It is interesting, further, to note that not all of the clinical manifestations of serum disease occur simultaneously, but that fever, adenopathy and urticaria usually precede the arthritis. In those rare cases in which there is a relapse of the serum disease the skin manifestations ordinarily differ from those seen in the first attack. The primary skin rash is practically always urticarial in nature; the second, if such occurs, is very finely macular or maculopapular, often situated about the hair follicles, apparently involving, therefore, some of the special organs of the skin rather than the skin as a whole. In an occasional case of the latter type which we have had the opportunity to study the precipitin titer at the time of the relapse has been distinctly higher than at the time of the first bout.

Opie (8) has shown that there is a rough parallelism between the intensity of the Arthus phenomenon and the concentration of precipitin in the sera of rabbits immunized either actively or passively to a soluble foreign protein. He concurs with the opinion, expressed

by others, that the inflammatory process is a reaction to an irritating compound which is formed by the local union of antibody and antigen. Several years ago one of us (9) showed that salicylate medication during the course of immunization partially inhibited antibody production in rabbits. The present observations seem to indicate that the two anti-rheumatic drugs used have a similar effect in human beings. If, however, all of the symptoms of serum disease were dependent upon the concentration of circulating antibodies it would be expected that drug treated patients would be practically free from any manifestation of the disease. Such is not the case; hence another explanation is required.

In recent years the view has been gaining ground that the cells of the reticulo-endothelial system play the chief rôle in the production of antibodies. Aschoff (10) and his co-workers have shown that not all of the cells of the body or even of the reticulo-endothelial system react similarly towards parenterally introduced particulate dyes or carbon particles. Certain groups of cells take up these particles readily, other groups do not react until after a more prolonged or intense exposure to the dye. Those of the skin stain very readily with trypan blue. It is probable that they absorb soluble proteins contained in foreign serum even more easily, and react by the production of antibodies. According to the theory of sessile and free antibodies a certain concentration of antibodies must be attained in the cells before they are set free into the tissue juices, and are detectable in the serum. Coördinating our observations with this theory we are led to the following explanation of the observed phenonena: The drugs so alter conditions that antibodies are discharged into the blood stream in very small amounts or not at all. This effect might follow either a lowered intracellular concentration or an altered permeability of the cell membrane. It seems, however, that antibodies must exist in the cells, for at a certain time the tissues give evidence of the presence of some irritating substance which probably results from the union in the cells of antigen and antibody, in sufficient concentration. In other words, the urticaria is an evidence of the active immunization of the reticulo-endothelial cells of the cutaneous tissue.

The fact that the arthritis in this disease appears later and practically only when there is a fairly high concentration of antibodies in the

serum, suggests that the cells of the articular tissue must be passively sensitized with antibodies before they are in a condition to show an inflammatory reaction. In other words, while the irritating substance that stimulates the inflammatory reaction may be the same in both the skin and joints, in the case of the former it is the result of active immunization of the cells, while in the latter it is the result of passive immunization. When for any reason this passive sensitization does not take place the patient remains free from arthritis. Support is given to this theory by the type of dermatitis observed in relapsing serum disease. Here, again, another type of cell seems to be involved than that taking part in the primary urticaria, and, as above mentioned, there is usually a concomitant high concentration of antibodies in the patient's serum. It is probable that the tissues of the skin involved in the relapse have been passively sensitized in the same manner as have those of the joints.

Another possibility must be considered. Dale and Hartley (11) have shown that when an animal is injected with a mixture of antigens the maximum time of immunization or sensitization may be different for each individual antigen. It is well established that serum contains several distinct antigenic proteins, and it is possible that the urticaria is attributable to a toxic antigen antibody complex involving one serum protein and the arthritis to a similar complex involving another. It is also possible that the depressing influence of the drugs on antibody formation is more powerful against the more slowly forming hypothetical arthrotropic antibody. If this were true one would expect with a complex antigen such as horse serum to demonstrate two curves or a curve with two peaks, one at the time of urticaria and another at the time of arthritis. Such a complex curve is rarely if ever found. We are, therefore, more inclined to the theory of active sensitization of the skin and passive sensitization of the joints as an explanation of the observed phenomena.

SUMMARY

1. If, immediately following the discontinuance of serum therapy, neocinchophen or aspirin in adequate dosage is given to patients and continued throughout the usual period of serum disease, arthritis is usually prevented even though other manifestations of serum disease occur.

2. The serum of patients treated in this manner usually fails to contain anti-horse serum precipitin, and only rarely shows a precipitin concentration above 1:40.

3. Usually a precipitin content of 1:400 is necessary before the patient shows arthritis.

4. The theory is advanced that urticaria in serum disease is the result of active sensitization of the skin which is not prevented by the drug treatment, while the arthritis is the result of passive sensitization of the joints which is inhibited when the circulating antibodies in the serum are kept to a low concentration by the anti-rheumatic drugs.

BIBLIOGRAPHY

1. Von Pirquet, C., and Schick, B., Die Serumkrankheit, Leipsic and Vienna 1905.
2. Boots, R. H., and Swift, H. F., J. A. M. A., 1923, lxxx, 12. The Arthritis of Serum Sickness.
3. Swift, H. F., J. Exper. Med., 1924, xxxix, 497. The Pathogenesis of Rheumatic Fever.
4. Hamburger, F., and Moro, E., Wien. klin. Woch., 1903, xvi, 445. Ueber die biologisch nachweisbaren Veränderungen des menschlichen Blutes nach der Seruminjektion.
5. Von Pirquet, C., and Schick, B., Die Serumkrankheit, Leipsic and Vienna, 1905.
   Lemaire, H., Recherches cliniques et expérimentales sur les accidents sérotoxiques, Thèse de Paris, 1906–1907.
   Wells, C. W., J. Infect. Dis., 1915, xvi, 63. On the Specific Precipitin in the Blood of Persons Injected with Antidiphtheric Horse Serum.
   Weil, R., Proc. New York Path. Soc., 1916, xvi, 68. The Analysis of Serum Sickness.
6. Longcope, W. T., and Rackemann, F. M., J. Exper. Med., 1918, xxvii, 341. The Relation of Circulating Antibodies to Serum Disease.
7. Mackenzie, G. M., and Leake, W. H., J. Exper. Med., 1921, xxxiii, 601. Relation of Antibody and Antigen to Serum Disease Susceptibility.
8. Opie, E. L., J. Immunol., 1924, ix, 231. Inflammatory Reaction of the Immune Animal to Antigen (Arthus Phenomenon) and its Relation to Antibodies.
9. Swift, H. F., J. Exper. Med., 1922, xxxvi, 735. The Action of Sodium Salicylate upon the Formation of Immune Bodies.
10. Aschoff, L., Lectures on Pathology, New York, 1924, Chapter I, Reticulo-Endothelial System.
11. Dale, H. H., and Hartley, P., Biochem. J., 1916, x, 408. Anaphylaxis to the Separated Proteins of Horse-Serum.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

V. THE ELEVATION OF TEMPERATURE IN THE PNEUMONIC LUNG.

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PLATES 34 AND 35.

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INTRODUCTION.

Two of us (C. B. and R. C.) have shown that the systemic temperature can be raised by diathermy, but that there is relatively little local heat developed in the normal lung (1). Thermocouples placed in the lobes of the lungs of anesthetized dogs seldom registered more than 0.4°C. higher than simultaneously recorded rectal temperatures. This is true in spite of the fact, now established (2), that the high frequency current produced by a diathermy machine actually penetrates the body and passes through the lungs, generating heat in the tissues it traverses. Why there is no marked local elevation of temperature in these tissues has been shown to be due to the fact that the heat generated is rapidly disseminated throughout the body by the circulating blood. The blood leaving the lungs can be shown to have been heated by the current (3). If, however, the pulmonary circulation to one lung is interrupted, a precipitous rise in temperature occurs in the ischemic lung (4). The degree of local heating which occurs under these circumstances will depend upon the extent of interference with the pulmonary circulation. This can be shown by interrupting the flow of blood through one branch of the pulmonary artery, leaving the veins patent and the bronchial circulation intact. There results from this procedure an increase of temperature in the ligated lung amounting to about 1.5°C. in excess of the temperature in the normal lung. The increase occurs immediately after the artery is clamped, while the subsequent rate of heating is similar to that in the control

lung. When, on the other hand, the veins to one lung are ligated, the local heating is far greater than under the conditions just described. Reference to the figures in Paper II of this series will bring out these facts.

From the foregoing, the implication seems obvious that a disease process accompanied by impairment of local circulation may be expected to provide conditions consistent with the production of local heat by the passage of high frequency currents through the region involved. That the pulmonary consolidation of pneumonia represents a disease process in which local circulatory impairment exists, there is evidence to believe. We cite in this connection the pathological studies of Kline and Winternitz (5), the injection preparations of Gross (6) and the inferences to be derived from analyses of the oxygen saturation of the arterial blood (7). The present study was undertaken for the purpose of discovering whether the consolidated lobe could in fact be heated above the temperature of the uninvolved, relatively normal lung tissue. The difficulties attending the production of experimental lobar pneumonia in laboratory animals are well known. Though the pathological lesions which presented themselves in the experiments about to be reported were not perhaps identical with those commonly seen in human lobar pneumonia, they, at least, may be said to simulate this condition and to represent a more or less complete consolidation of a lobe, or the major part of it.

We have found that such a consolidated lobe can be heated by a diathermy current of the strength generally used in therapy to a point approximately 1°C. to 2°C. above the temperature of the surrounding normal lobes.

Methods.

Dogs were used in all of these experiments. During deep ether anesthesia they were inoculated by the method of intrabronchial insufflation described by Lamar and Meltzer (8). Actively growing broth cultures of two varieties of organisms were used: *B. friedlænderi* Type B and Pneumococcus Type I. An effort to maintain virulence was made in the case of Friedländer's bacillus by passage through guinea pigs, and in the case of pneumococcus by mouse passage. The cultures were injected intrabronchially in volumes varying from 1 to 3 cc. per kilo body weight. Even with careful control of conditions the results of these inoculations were uncertain and variable. Some animals died within 24 hours of inoculation, before the appearance of a pulmonary lesion. Others survived ap-

parently unharmed. It was necessary to select animals for use on the basis of x-ray evidence of pneumonic consolidation.

The method of lung temperature measurement and diathermy administration was the same as that previously described (1). Observations were made on the dogs after they had been anesthetized by the intravenous injection of a 10 per cent solution of barbital-sodium, the dose not exceeding 0.3 gm. per kilo body weight. The low initial temperatures often observed may be ascribed in part to the result of infection, in part to heat loss resulting from anesthesia. This was difficult to prevent, since we thought it best to use no hot pad or source of heat other than diathermy. Considerable practice was necessary before we learned to lodge the thermocouple needle in the substance of the consolidated lobe. Owing to the differences in the shapes and sizes of the dogs it was difficult to find any external landmarks which would act as guides for the insertion of the needle into any particular area. In the majority of instances the lower lobe alone was consolidated. In order to get a thermocouple into it the needle was usually inserted close to the spinal column, through the 9th or 10th intercostal space. Even with this guide the element of luck remained a large one. Other thermocouples were inserted in symmetrical positions into the opposite healthy lobes. At the close of each experiment an autopsy was done in which the positions of all thermocouples were located. The gross appearance of the lungs was described in relation to the site of the thermocouples and the lungs were usually saved either for injection preparations or for microscopic examination.

EXPERIMENTAL.

Experiment D 36.—May 19, 1927. At 2:45 p.m. a male mongrel fox terrier weighing 12.7 kilos was given ⅛ gr. morphine sulfate subcutaneously. About 15 minutes later it was etherized deeply. A short piece of rubber tubing was passed through the larynx into the trachea, and through this was inserted a soft rubber tube 30 cm. long, with an outside diameter of 3 mm. When the bronchial catheter was in place its proximal end was connected with the nozzle of a record syringe previously filled with 26 cc. of a 4 hour broth culture of *B. friedlænderi*. The whole mass of culture was then injected and forced into the lung with a small quantity of air. There was no cough following injection. The animal made a quick recovery from the anesthetic.

May 20, 12, noon. The dog looked moderately sick. The respirations were rapid, but not labored. Rectal temperature was 39.5°C.

May 21, 9 a.m. The dog was now definitely sick. An x-ray photograph showed a slight shadow at the base of the left lung (Fig. 1).

May 21, 10 a.m. The animal was anesthetized by the intravenous injection of 27 cc. of a 10 per cent solution of barbital-sodium.

10:30 a.m. The sides of the dog's thorax were shaved and lead-tin electrodes 3 × 4 inches square were applied to the shaved skin. To assure perfect contact several layers of gauze moistened with a solution made from mixing equal parts of

glycerol and saturated saline were interposed between skin and electrodes. A rectal thermometer was put in place and the thermocouples were thrust through the chest wall into the lungs.

11:10 a.m. The larynx was intubated to facilitate breathing.

11:14 a.m. to 1:50 p.m. Thermocouple readings were made at 10 to 15 minute intervals (Table I). The diathermy current was turned on at 11:29 a.m. and ran continuously until 1:50 p.m.

1:55 p.m. The dog was killed by the intravenous injection of 20 cc. of a saturated solution of $MgSO_4$, the trachea being clamped in inspiration.

The thorax was opened and the position of the thermocouples and condition of the lungs established.

Thermocouple 1 was buried in the substance of the lower portion of the left upper lobe, near the lung root. It was lying in a discolored, congested area of the lobe which was, however, air-containing.

Thermocouple 3 had transfixed the upper pole of the left lower lobe and lay in that portion of the ventral lobe which adjoins the hilum. This part of the lobe was dark and definitely abnormal in appearance, as was the left lower lobe immediately adjoining it, which was purplish, boggy and non-air-containing. No thermocouple was found in this left lower lobe.

Thermocouple 2 was found in the substance of the right upper lobe in what was apparently normal lung tissue.

Thermocouple 4 lay in the pleural cavity projecting into the interlobar space and surrounded by healthy tissue.

To summarize the situation found at autopsy: Two of the thermocouples, Nos. 1 and 3, were found within the substance of the left lung, which was the seat of a pneumonic process. Although lying in pathological tissue, neither had penetrated the lower lobe, where the consolidation was most pronounced. The other two thermocouples, Nos. 2 and 4, lay in, or in close proximity to, the normal tissue of the right lung.

As to the temperature changes recorded by these thermocouples: Both thermocouples, Nos. 1 and 3, showed throughout the duration of current flow a greater rate of heating than Nos. 2 and 4, which were lodged in the opposite normal lung. The rate was greater in No. 3 than in No. 1, corresponding in this to the degree of structural damage found at the respective sites of these thermocouples. Comparing the temperatures developed in the left lung and right lung (Table I, Text-fig. 1) one sees, for example, in Thermocouple 2 (right healthy lung) a rise of 4.52°C. in 2 hours and 21 minutes of diathermy. The rectal temperature during this period had risen 4.42°C., while Thermo-

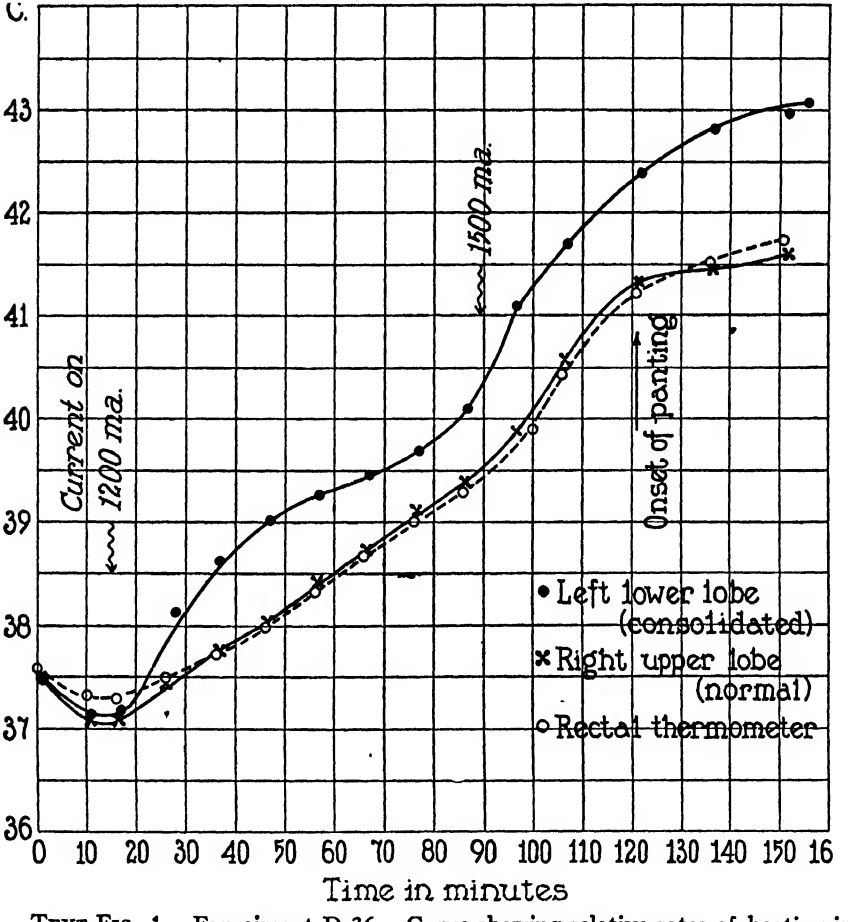
TABLE I.

Temperature Changes Recorded in Experiment D 36.

Time	Current	Rectal thermometer	Thermocouple 1 in left lung (pathological)	Thermocouple 2 in right lung (normal)	Thermocouple 3 in left lung (pathological)	Thermocouple 4 in right lung (normal)	Thermocouple 5 in subcutaneous tissue under left electrode	Thermocouple 6 on skin under left electrode	Thermocouple 7 on skin under right electrode	Pulse rate per min.	Respiratory rate per min.	Remarks
	ma.	°C.										
11:14	0	37.57*	37.50	37.48	37.48	37.51	35.98	35.17	36.07	29	166	Respirations irregular
11:24	0	37.31	37.19	37.08	37.14	37.16	35.98	35.07	36.00	37	168	
11:30	1200	37.28	37.33	37.09	37.68	37.20	47.78	38.12	39.15	38	168	Current on at 11:29
11:40	1200	37.48	37.62	37.42	38.13	37.43	50.13	41.40	41.30	28	164	
11:50	1200	37.71	37.94	37.75	38.62	37.74	52.11	43.31	42.71	31	166	
12:00	1200	37.98	38.26	38.01	39.01	38.05	51.87	43.88	42.86	30	164	
12:10	1200	38.32	38.68	38.41	39.26	38.43	50.68	42.85	42.48	24	170	
12:20	1200	38.66	38.92	38.72	39.46	38.75	50.77	42.88	42.45	23	172	
12:30	1200	39.00	39.18	39.12	39.69	39.16	50.52	42.98	42.45	23	180	
12:40	1500	39.29	39.52	39.39	40.10	39.41	50.49	43.16	42.55	23	184	Current increased to 1500 ma. at 12:43½
12:50	1500	39.89	40.14	39.88	41.09	39.99	56.30	45.29	44.38	25	186	Rectal temperature read at 12:54
1:00	1500	40.43	40.84	40.57	41.69	40.68	55.72	45.92	44.81	24	188	
1:15	1500	41.21	41.70	41.32	42.38	41.46	55.00	45.92	45.05		190	Panting began suddenly at 1:16
1:30	1500	41.52	42.07	41.45	42.82	41.60	54.65	45.88	45.32		194	Panting.
1:45	1500	41.73	42.25	41.60	42.98	41.75	54.12	45.88	45.29		204	Panting.
1:50	1500				43.08							Current off at 1:50½
1:50¼	0				42.51							
1:50½	0				42.32							

*The rectal thermometer and Thermocouple 1 were read at the times indicated. Other readings were made at ½ minute intervals.

couple 3, in diseased lung, showed an increase of 5.84°C., or 1.32°C. above the temperature developed in the control lung. Such an increase corresponds in order of magnitude to the change shown to occur



TEXT-FIG. 1. Experiment D 36. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature. After 107 minutes of current flow the dog suddenly began to pant. This resulted in cooling of the normal lung, which thereupon fell below the rectal temperature. No such change occurred in the pathological lobe.

during diathermy in a lung after clamping its pulmonary artery (4). The experiment just described (D 36) was repeated in six other dogs. Because of the inconstant pathological changes produced by Fried-

länder bacillus insufflations, and because of the inherent uncertainty of directing the thermocouple needles into both consolidated and healthy lung tissue, it was impossible to demonstrate heating of the pathological lobe in all experiments. We have deliberately chosen for publi-

TABLE II.

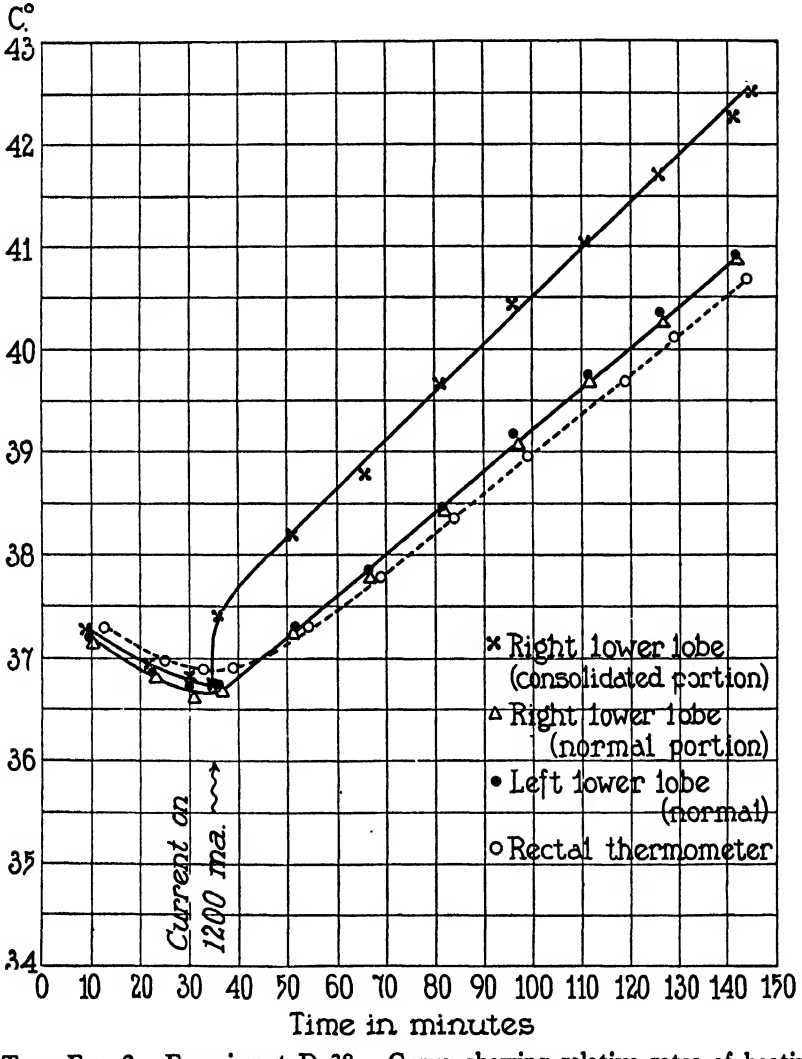
Temperature Changes Recorded in Experiment D 38.

Time	Current	Rectal thermometer	Thermocouple 1 in right lung (consolidated)	Thermocouple 2 in left lung (normal)	Thermocouple 3 in right lung (pathological)	Thermocouple 4 in left lung (normal)	Thermocouple 5 in right lung (pathological)	Thermocouple 6 on skin under right electrode	Thermocouple 7 on skin under left electrode	Pulse rate per min.	Respiratory rate per min.
	ma.	°C.									
11:39	0	37.30*	37.29	37.20	37.18	37.18	37.29	34.52	34.76		48
11:52	0	36.98	36.94	36.86	36.82	36.84	36.92	34.60	34.76	184	52
12:00	0	36.89	36.82	36.73	36.62	36.68	36.77	34.80	34.80		64
	On										
12:05	1200		36.75								
12:06	1200	36.90	37.42	36.75	36.68	36.82	36.90	37.13	38.11	208	64
12:21	1200	37.31	38.20	37.31	37.25	37.44	37.46	40.78	41.02	200	62
12:36	1200	37.79	38.77	37.85	37.79	37.96	38.07	42.90	42.68	200	56
12:51	1200	38.37	39.67	38.49	38.44	38.60	38.70	43.59	43.14	200	50
1:06	1200	38.96	40.43	39.18	39.07	39.28	39.35	43.71	43.38	196	46
1:21	1200	39.07	41.01	39.77	39.67	39.90	39.98	43.75	43.83	196	44
1:36	1200	40.12	41.70	40.37	40.27	40.50	40.61	44.06	44.29	196	46
1:51	1200	40.69	42.25	40.90	40.86	41.10	41.26	44.73	44.81		
	Off										
1:55			42.51								
1:55½	0		41.89								
1:55½	0		41.77								
1:55¾	0		41.70								
1:56½	0			41.02							

* The rectal thermometer and Thermocouple 1 were read at the times indicated. Other readings were made at ½ minute intervals.

cation in detail those experiments in which a certain degree of local heating was produced. It should be emphasized that failure to find local heating could always be accounted for on the basis of no consolidation or malposition of thermocouples, except in one instance, Experiment D 49, to be referred to below.

Experiment D 38.—On May 25, 1927, at 2:45 p.m., a male mongrel weighing 12.6 kilos, after a preliminary subcutaneous injection of ¼ gr. morphine sulfate, was given, under deep ether anesthesia, an intrabronchial insufflation of 25 cc.



TEXT-FIG. 2. Experiment D 38. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature.

of a 4½ hour culture of *B. friedlænderi.* The next afternoon at 4:20 the dog looked moderately sick and showed a rectal temperature of 40.1°C. An x-ray photograph showed a slight shadow at the base of the right lung (Fig. 2). On

May 27, at 9:30 a.m., the dog was coughing occasionally, but looked less sick than on the previous day. The animal received an intravenous injection of 37 cc. of a 10 per cent solution of barbital-sodium. When a satisfactory state of anesthesia had been achieved, electrodes were applied and thermocouples inserted in the usual manner. A current of 1200 milliamperes was passed through the thorax for 1 hour and 50 minutes. Immediately after the current was turned on it was found that Thermocouple 1 which had been directed into the right lower lobe through the anterior chest wall was recording a more rapid rise in temperature than any of the others within the thorax or than the rectal thermometer. This thermocouple, No. 1, was found at autopsy to be buried in the right lower lobe which had the appearance of liver, except for an air-containing portion on its anterior aspect. Both of the other thermocouples in the right lung were found in the upper portion of the lower lobe where the consolidation was less marked. The lesion on the right side increased in severity from above downward, which may perhaps account for the fact that the final temperatures observed in the right lower lobe showed a gradation from above downward: 40.86°C., 41.26°C., 42.25°C. The final temperature recorded by this thermocouple represented a rise of 5.45°C. in 1 hour and 50 minutes (Table II, Text-fig. 2), as compared with a rise of 4.17°C. and 4.42°C. respectively in thermocouples, Nos. 2 and 4, located in the normal left lower lobe. The increase in rectal temperature during the same interval amounted to 3.80°C. The final temperature of the skin under the electrodes was 44.73°C. on the right side, 44.81°C. on the left. The surface temperature cannot, therefore, be held responsible for the observed difference in internal temperature.

The findings in Experiment D 38 are quite comparable to those in D 36. In interpreting them the many controls supplied by our previous studies should be borne in mind, where it was clearly shown that the normal lung with intact circulation could be heated to an average maximum of 0.35°C. above the rectal temperature. In only one experiment of 13 did the temperature in the lung mount as high as 0.6°C. above the rectal during the course of diathermy. Since this animal died unaccountably while the experiment was in progress it is probable that the slight increase in heating can be explained on the basis of a failing pulmonary circulation.

Experiment D 48.—The procedure outlined in the previous experiments was again carried out in a female mongrel weighing 8.8 kilos. While etherized it received, on Nov. 6, 1927, 10 cc. of a *B. friedlænderi* culture intrabronchially. The next afternoon lung temperature measurements were recorded during diathermy. Autopsy showed the left lower lobe to be hepatized, while the upper and ventral lobes were air-containing and normal in appearance. The right lung was normal except for congestion and nodular consolidations along the posterior

margin of the lower lobe, most marked near the hilum. The caudal lobe, as well, was congested. The most marked temperature increase was recorded by one of the two thermocouples in the left lower hepatized lobe which showed a final rise of 1.62°C. above the rectal temperature. A second thermocouple in this lobe showed an increase of only 0.6°C. above the rectal, similar to the change observed in the thermocouple in the opposite lobe.

In this experiment a careful histological examination of the lungs was made particularly with an eye to finding evidence of circulatory obstruction. Such evidence was indeed found. The microscopic examination was made independently by one of us (W. E.) who was not informed of the other experimental findings.

Result of Microscopic Examination of Lungs in Experiment D 48.—(Figs. 4 and 5.) The lungs were distended with Zenker's fluid and immersed in it. Sections were taken from all lobes and imbedded in paraffin. These were cut to a thickness of 5 to 6 micra and stained with eosin-methylene blue, iron-hematoxylin-eosin and Gram's stain. The findings are given below:

Right upper lobe: The section appears normal.

Right ventral lobe: The section appears normal.

Right lower lobe: One section appears normal. A second, however, at its center shows certain pathological changes. The bronchioles and alveoli are uniformly filled with an exudate consisting of alveolar epithelial cells and polymorphonuclear leucocytes. The epithelial cells are often filled with vacuoles and with Gram-negative bacilli, often also with polymorphonuclear leucocytes and erythrocytes. There is everywhere a proliferation of young epithelial cells from the alveolar walls and polymorphonuclear leucocytes are to be seen within the walls of the bronchioles and bronchi. The walls of the blood vessels are edematous in places. In the smaller vessels the adventitia frequently shows a proliferation of round cells. The capillaries are dilated with blood and the lymph vessels of the pleura are dilated with lymph. The exudate diminishes toward the peripheral portions of the section where the alveoli appear normal.

Left upper lobe: In the center of the section there is an area similar to the one just described, but smaller.

Left lower lobe: In three sections one sees the alveoli and bronchioles uniformly filled with exudate. There are no air-containing ones to be seen. The exudate has the same composition as described in the right lower lobe but is apparently greater in quantity. In some places where the exudate is plentiful there are comparatively few old, mostly phagocytosed erythrocytes. Here the capillaries are compressed and contain no red blood corpuscles (Fig. 5). Where there is less exudate, well preserved erythrocytes and some fibrin are often to be seen lying in the alveolar spaces. Here the capillaries are dilated with blood. The walls of the large vessels are edematous and infiltrated with many erythrocytes. The epithe-

lial lining of the bronchioli is mostly desquamated, that of the larger bronchi is well preserved. Many of the bronchial walls exhibit hemorrhages. The lymph vessels are dilated, containing erythrocytes, polymorphonuclear leucocytes and fibrin.

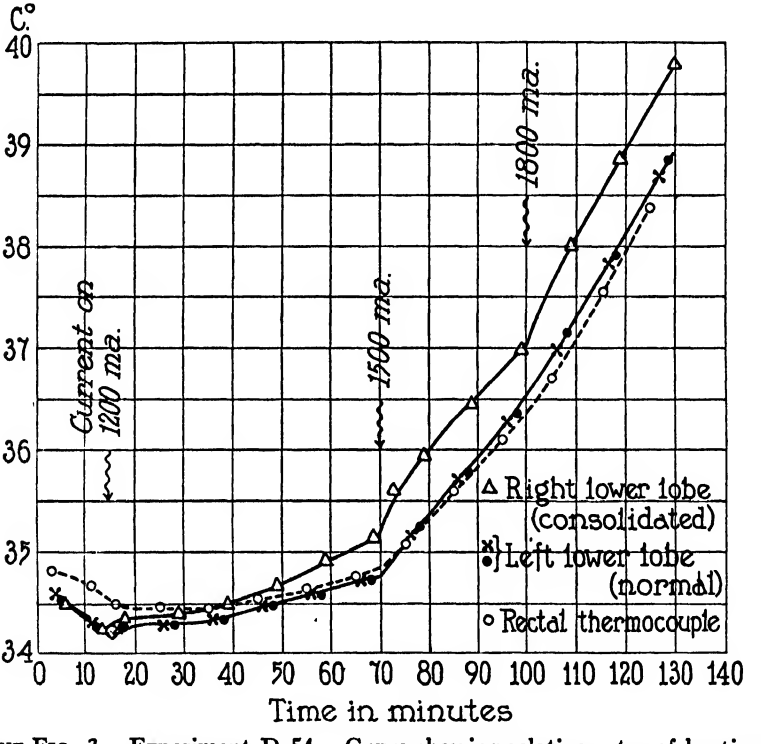
The significant finding is the compressed empty capillaries. Apparently this is due to the pressure of the exudate and depends for its occurrence upon the amount of exudate. This ischemic condition was found only in certain parts of the left lower lobe. One of the two thermocouples in this lobe showed a rise of temperature greater than that occurring in any of the other lobes. The second thermocouple, however, recorded an increase in temperature equal to that in the right lower lobe. The experimental findings are then quite consistent with the changes observed by microscopic study.

In Experiment D 49 the dog received 1 cc. per kilo of an actively growing culture of *B. friedlænderi*. Observations were made the following day. The dorsal half of the right lower lobe was consolidated. There was no local heating recorded by either of the two thermocouples imbedded in the pathological lobe. The final temperatures recorded in the consolidated portion of the right lower lobe were 37.75°C. and 37.87°C. after 42 minutes of diathermy at 1200 milliamperes, as compared with a temperature of 37.97°C. in the relatively normal left lower lobe. The rectal temperature recorded simultaneously by a mercury thermometer was 37.68°C. In this animal the microscopic picture of the right lower lobe (Fig. 6) showed the alveoli to be very irregularly filled with exudate. Air-containing alveoli were seen lying between those showing the inflammatory reaction. The capillaries, far from being compressed and empty, were dilated with blood. A similar dilatation of vessels, but no exudate, was found in the left lower lobe. It seems not improbable that the lack of local heating was associated with the absence of vascular obliteration.

A number of experiments was made, using cultures of Pneumococcus Type I in place of *B. friedlænderi* for intrabronchial insufflation. It was hoped that the fibrin reaction, usually stimulated by Type I pneumococcus, would cause a greater interference with the pulmonary circulation and therefore permit of more definite local heating. This was not the case. The response of dogs to intrabronchial insufflations of Type I pneumococci is an extremely variable one. They will often

tolerate large amounts of actively growing mouse-virulent cultures when injected by this route. We have selected for publication one experiment (D 54) in which a pneumonic consolidation was produced involving the caudal lobe and three-quarters of the right lower lobe, where a thermocouple had been placed.

The cut section of the right lower lobe was solid and apparently non-air-containing. Histological examination of the right lower lobe showed the alveoli and bron-



TEXT-FIG. 3. Experiment D 54. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature.

chioles mostly filled with exudate. In some places air-containing alveoli occurred amongst the others. The exudate consisted chiefly of polymorphonuclear leucocytes mixed with varying numbers of vacuolated alveolar epithelial cells often containing erythrocytes and degenerated cocci. Most of the capillaries were dilated with blood. Red cells were seen in the alveolar exudate and in some places hemorrhages were present in the walls of the bigger vessels. No evidence of capillary fibrin thrombi was to be found. The left lung was apparently quite normal though sections from the lower lobe showed that the vessels were dilated

with blood. Difference in temperature between the pathological and normal lobes was slight, but became more apparent on increasing the milliamperage to 1800 (Text-fig. 3). The final temperature in the consolidated lobe was 1°C. above the rectal temperature while the normal lung was 0.15°C. above the rectal temperature. The slight degree of elevation of temperature in the involved lobe produced by the current of 1200 milliamperes may be explained on the basis of no clear evidence of a grossly impaired circulation, since neither capillary thrombi were present nor sufficient exudate to compress the capillaries. For the higher current strengths it is probable that the existing circulatory disorder was sufficient to permit of a moderate degree of local heating.

The existence of obstruction to the circulation was further indicated by injection preparations made with the technique described by Gross (6). Satisfactory preparations could not be obtained at each attempt. The final picture depended upon the conditions prevailing during injection. The presence of puncture holes caused by the thermocouple needles made it impossible to use the lungs, in most of these experiments, for purposes of injection.

We present in Fig. 3 the photograph of a cleared dog's lung injected immediately after death with a barium-gelatin mixture. 2 days before, the animal (D 35) had been injected intrabronchially with 25 cc. of a broth culture of *B. friedlænderi*. It was killed by injecting 20 cc. of a saturated solution of $MgSO_4$ intravenously. The area of involvement described in the autopsy notes corresponds exactly to that portion of the lung which has injected poorly. We publish this photograph as further evidence for the impairment of the circulation in the pneumonic lung.

DISCUSSION.

We have deliberately chosen for publication only those experiments in which the proper combination of circumstances made it possible to demonstrate local heating of the lung by diathermy. These circumstances consist first of the development of a pulmonary lesion sufficient to cast a shadow when photographed by x-ray, and secondly of the accurate locating of thermocouples in both consolidated and normal lung tissue. When these conditions have been fulfilled we have been able to demonstrate local heating in three of four animals inoculated intrabronchially with cultures of *B. friedlænderi* and in one animal inoculated in a similar fashion with a culture of Pneumococcus

Type I. The variation of heat production in these selected experiments can be accounted for by the positions of the thermocouples and by the degree of impairment of the pulmonary circulation. The pathological process in these dogs' lungs presents three stages: (1) a stage of active congestion (Fig. 6) in which relatively little exudate is to be seen and the alveolar capillaries are dilated with blood; (2) a transitional stage (Fig. 4) in which the amount of exudate is increased and the erythrocytes lying in the capillaries have lost their regular shapes and usual staining properties, and finally (3) an ischemic stage (Fig. 5) in which all the alveoli are uniformly filled with exudate and the capillaries are compressed and free from erythrocytes. This final bloodless stage in which the local circulation is definitely impaired is the one in which we believe a certain degree of local heating with diathermy may occur.

We have purposely drawn no inferences as to heat production during diathermy in the lung of a patient suffering from pneumonia.

SUMMARY AND CONCLUSIONS.

1. An experimental pneumonia with more or less lobar distribution has been produced in dogs by the method of intrabronchial insufflation of *B. friedlænderi*, Type B, and Pneumococcus, Type I.

2. Such dogs as showed evidences of a pulmonary lesion when photographed by x-ray were selected for lung temperature measurements.

3. Measurements of lung temperature were made by means of thermocouples before and during diathermy.

4. The thermocouples which recorded the temperature in the consolidated lobes showed in most instances a more rapid rate of heating during diathermy than those in the normal lobes. The final increase in temperature in the pathological lobes over the normal lobes amounted to slightly more than 1°C.

5. When local heating occurred during diathermy it was of the order of magnitude found in a lung in which the branch of the pulmonary artery supplying it had been clamped.

6. Histological examination of the lungs showed the pathological reaction to consist of intraalveolar exudate composed of polymorphonuclear leucocytes and desquamated alveolar epithelium. In some

sections the exudate was sufficient to cause compression and emptying of the alveolar capillaries.

7. The local heating, we believe, depends upon this ischemic state of the smaller vessels.

8. Further evidence for an imparied circulation in the pneumonic lung is furnished by injection preparations in which the uninjected area corresponded exactly to the gross pathological lesion.

BIBLIOGRAPHY.

1. Binger, C. A. L., and Christie, R. V., *J. Exp. Med.*, 1927, xlvi, 571.
2. Christie, R. V., and Binger, C. A. L., *J. Exp. Med.*, 1927, xlvi, 715.
3. Binger, C. A. L., and Christie, R. V., *J. Exp. Med.*, 1927, xlvi, 595.
4. Binger, C. A. L., and Christie, R. V., *J. Exp. Med.*, 1927, xlvi, 585.
5. Kline, B. S., and Winternitz, M. C., *J. Exp. Med.*, 1915, xxi, 311.
6. Gross, L., *Canad. Med. Assn. J.*, 1919, ix, 632; *J. Med. Research*, 1917, xxxi, N.S., 327.
7. Lundsgaard, C., and Van Slyke, D. D., Cyanosis, Medicine monographs, Baltimore, 1923; *Medicine*, 1923, ii, 1.
8. Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. X-ray photograph of thorax of Dog D 36 taken 42 hours after intrabronchial insufflation with culture of *B. friedlænderi*. The picture shows a slight shadow at the base of the left lung.

FIG. 2. X-ray photograph of thorax of Dog D 38, taken 26 hours after intrabronchial insufflation with culture of *B. friedlænderi*. The picture shows a slight shadow at the base of the right lung.

FIG. 3. Experiment D 35. Photograph of cleared barium-gelatin injection preparation of dog's lung in which consolidation had been produced by the intrabronchial insufflation of a culture of *B. friedlænderi*. The uninjected area corresponded to the part of the lung which showed gross pathological changes at autopsy.

PLATE 35.

FIG. 4. Experiment D 48. Left lower lobe. Magnification × 400. The photograph shows the transitional stage in which the erythrocytes in the capillaries have become irregular in shape and have lost their usual staining properties. Methylene blue and eosin.

FIG. 5. Experiment D 48. Left lower lobe. Magnification × 400. This represents the final ischemic stage. All the pulmonary alveoli are uniformly filled with exudate, the capillaries being compressed and bloodless. Methylene blue and eosin.

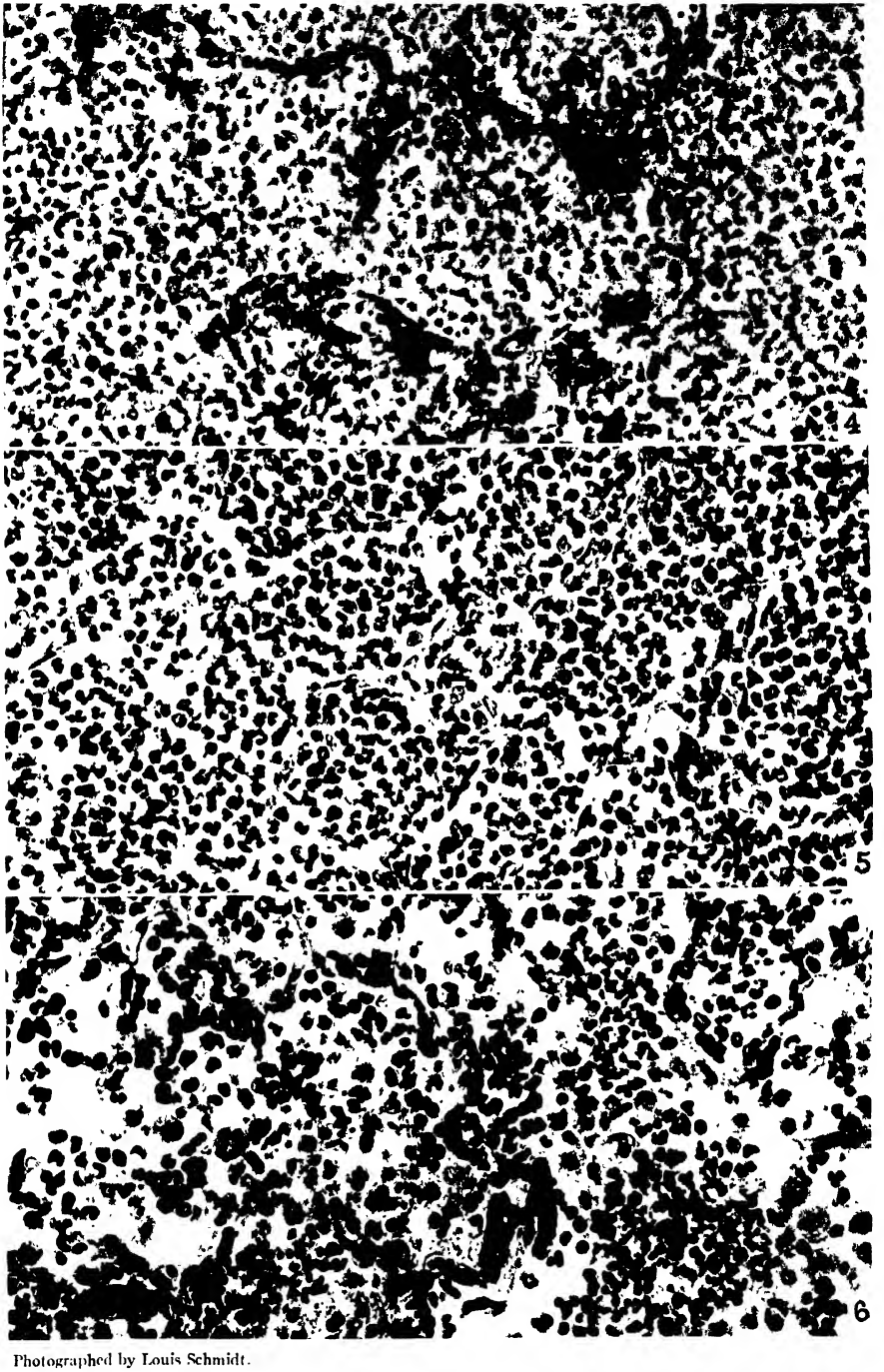
FIG. 6. Experiment D 49. Right lower lobe. Magnification × 400. The photograph represents the stage of active congestion in which the alveolar capillaries are dilated with blood. Methylene blue and eosin.

1

2

3

(Christie, Ehrich, and Binger: Diathermy. V.)



Photographed by Louis Schmidt.

(Christie, Ehrich, and Binger: Diathermy. V.)

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

IV. ANAPHYLAXIS WITH TWO NON-TYPE-SPECIFIC FRACTIONS.

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Anaphylactic experiments with bacterial proteins have presented more difficulties to investigators in this field than similar experiments with proteins from other sources. This has probably been due, to a certain extent, to the primary toxicity of many bacterial proteins and, in part, to the difficulty of obtaining a sufficient quantity of concentrated material.

Zinsser and Parker (1), working with a slightly alkaline saline extract of typhoid bacilli, noted its primary toxicity and the quantitative rather than the qualitative differences between passively sensitized and normal guinea pigs when relatively large amounts of antigen were injected; whereas with smaller amounts, they did not obtain acute anaphylactic death. Using the isolated uterus method of Dale (2), they were able to eliminate the error due to toxicity of the antigen, since normal uteri did not react with typhoid antigen. They showed, in this way, that the reactions obtained with bacterial antigens were essentially similar to those obtained with such antigens as horse serum. A critical review of the previous literature on bacterial anaphylaxis is given by these authors.

The experiments reported in this and in the succeeding paper were undertaken primarily to throw some light on the question of the antigenicity of the various fractions isolated from the hemolytic streptococcus. It had already been shown that certainly two and possibly three distinct reactive substances, all non-type-specific, were present in extracts of hemolytic streptococci in addition to the type-specific protein, and that these could be separated chemically and serologically (3). Certain points of interest soon arose with regard to the anaphylactic reactions caused by these fractions, and these observations are recorded. The results presented in this paper were

obtained with the group-reactive nucleoprotein (P) and with the species-specific substance (C) which is probably a carbohydrate; while the results with the type-specific protein (M) and the non-type-specific protein (Y) sometimes associated with it are given in the succeeding paper.

Methods.

The preparation of the extracts used in these experiments has been previously described (3). Briefly, the nucleoprotein (P) was the fraction of NaOH extracts precipitable in the cold with dilute acetic acid, while the probable carbohydrate (C) was obtained from the supernatant fluids after precipitation of the other reactive fractions from their respective extracts. It was freed of protein as far as the small yields permitted. The type-specific (M) protein was obtained from HCl extracts.

The preparation of antibacterial sera was described in detail in a preceding paper and the most satisfactory method of preparing anti-P sera, also described previously (3), is summarized here for convenience. Rabbits were immunized with P by intravenous injections of 1 per cent solutions. Four daily injections of 10 mg. each were made, followed by a 3-day rest, then four more injections of 20 mg. each followed by 3 days of rest as before. A similar third series of injections of 30 mg. doses was given, then a fourth series of 40 mg. doses. If the titer of the test bleeding, taken 7 days later, was unsatisfactory, further series of 40 mg. doses were made until a satisfactory titer was obtained. The animal was then bled and the serum stored in the ice box without preservative.

Guinea pigs were sensitized passively by the intraperitoneal injection of 0.5 cc. of immune serum. Occasionally larger amounts of serum were employed and are thus recorded in the tables. Since it was impossible to obtain enough guinea pigs of exactly the same weight for use in all experiments, animals of approximately the same weight were selected for each experiment. The weights, which usually ranged between 150 gm. and 200 gm., are omitted from the tables. The sensitized animals were tested by intravenous injections of the appropriate extracts at varying intervals after sensitization, as shown in the tables. The smallest amount of extract which regularly caused acute anaphylactic death was called the minimal anaphylactic dose (M. A. D.), following the terminology of Weil (4). Since the concentration of active substances in the extracts was sometimes unknown, the dose could not always be expressed in mg. and was, therefore, in these instances given as M. A. D. for guinea pigs sensitized with the homologous serum. Sometimes both methods of recording the dosage were employed. In those instances in which it was desirable to test the anaphylactic reactions of a sensitized guinea pig with more than one substance, it was found necessary to wait 24 hours between injections in order to give the animal time to recover from the non-specific reduction of reactivity due to the first shock. The necessity for this interval has also been noted by Wells and Osborne in their study of the

relationships of certain plant proteins by means of the anaphylactic reaction (5). Control animals were always injected with doses at least as large as those used for the sensitized guinea pigs, sometimes following an intraperitoneal injection of normal rabbit serum and sometimes without this preliminary injection. They never showed symptoms of shock with the doses employed and survived indefinitely, most of them being kept under observation for several weeks.

A convenient method for making intravenous injections into guinea pigs was brought to our attention by Dr. C. H. Hitchcock. The hair was shaved from the back of the leg and a superficial longitudinal vein was easily laid bare by a small incision in the skin just above the ankle. This vein was small but was satisfactory for intravenous injections with a 25 gauge Yale needle. As many as five separate injections have been made into the same vein, although usually the veins in both legs were used for repeated injections, and occasionally similarly located veins in the forelegs were employed. All intravenous injections reported here were made in this manner.

The results of anaphylactic tests of each reactive fraction injected into guinea pigs sensitized with anti-P and with antibacterial sera are considered in order. The group-reactive P antigen is discussed first since a pure anti-P serum was available for its study. Since Zinsser and Parker reported optimal sensitization after an interval of 5 to 8 days rather than earlier, the following experiment was performed to determine the optimal time for the shocking injection after passive sensitization with this serum.

Experiment 1.—A series of guinea pigs was sensitized with anti-P serum.[1] The M. A. D. of the homologous P was determined at 24-hour intervals throughout a 4-day period. The results are tabulated in Table I.

The experiment showed that the optimal time for passive sensitization with this anti-P serum was not reached until the 2nd or 3rd day, as judged by the size of the M.A.D., and that guinea pigs became less sensitive after the 3rd day.

The next experiment was devised to show the relationships among nucleoproteins from different types of hemolytic streptococci as well as from *Streptococcus viridans* and pneumococcus.

Experiment 2.—Another series of guinea pigs was sensitized with anti-P serum. The M. A. D. of nucleoprotein from strains representing three different types of

[1] In each instance the details of preparation of immune serum are given in the tables; hence they will not be discussed in detail in the text.

hemolytic streptococcus was determined on the day after sensitization by the intravenous injection of varying amounts of a 1 per cent solution. Sensitized

TABLE I.

Anti-P Reactions.

Time Required for Passive Sensitization with an Anti-P Serum.

Guinea pigs sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, *Type* S23.

Shocked by intravenous injections of P from homologous strain, S39			
Guinea pig	Days after sensitization	Dose	Result
		mg.	
A-1	1	5.0	+++++
A-2	1	7.5	†5 min.
A-3	2	0.5	+
A-4	2	1.0	†4 min.
A-5	3	0.5	±
A-6	3	1.0	++
A-7	3	2.0	†5.5 min.
A-8	4	2.0	+
A-9	4	5.0	†3.5 min.

20 mg. of P did not shock unsensitized control guinea pigs.

In all tables the following symbols are employed:

− indicates no shock.
±? " trace of shock.
± " slight shock.
+ " mild shock.
++ " moderate shock.
+++ " moderately severe shock.
++++ " severe shock.
+++++ " very severe shock.
† " animal died.

hom. = homologous.
het. = heterologous.
M. A. D. = minimal anaphylactic dose.

guinea pigs were also tested with P from a strain of green streptococcus and with P from pneumococcus, Type III.[2] Animals surviving the first injection were

[2] This protein was an oxidized extract kindly supplied by Dr. Julianelle.

reinjected on the following day with a dose of hemolytic streptococcus P several times the M. A. D. in order to determine whether they had become desensitized. Control animals were not shocked by 20 mg. of P.

TABLE II.

Anti-P Reactions.

Minimal Anaphylactic Dose of Nucleoprotein, P, from Different Strains.

Guinea pigs passively sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, *Type* S23.

Guinea pig	Shocked by intravenous injections					
	First test 1 day after sensitization			Second test 2 days after sensitization		
	Antigen: P from strain of *S. hæmolyticus*	Dose	Result	Antigen: P from strain of *S. hæmolyticus*	Dose	Result
		mg.			mg.	
B-1	S39 (hom.)	5.0	++	S39 (hom.)	10*	±
B-2	" "	7.5	†3 min.			
B-3	S43 (het.)	5.0	+	" "	10	±?
B-4	" "	7.5	†4.5 min.			
B-5	S3 "	5.0	++**			
B-6	" "	7.5	†4.5 min.			
	P from strains of related species					
B-7	V92 (*S. viridans*)	10.0	+	" "	10	†4 min.
B-8	" " "	15.0	++	S3 (het.)	20	+++
B-9	" " "	20.0	+++	S39 (hom.)	10	++++
B-10	Pneumococcus, Type III	2cc.***	†4 hrs.			

* 10 M. A. D. for 48 hours. See Table I.

** Found dead next day. Small Gram-negative bacillus in heart's blood culture.

*** Concentration of P unknown. Test made 48 hours after sensitization. A control animal was unaffected by the same dose.

Table II shows the results of this experiment. Although the three hemolytic streptococcus proteins represented three distinct serological types, the M.A.D. for guinea pigs sensitized with Serum R500 was the same; while shock, but not death, was produced by considerably

larger doses of green streptococcus protein. A pneumococcus protein injected 48 hours after sensitization resulted in delayed death. Animals surviving shock from the hemolytic streptococcus proteins were completely protected against shock on the following day with 10 mg. of homologous P, although with this interval after sensitization 1 mg. was the M.A.D.; and those surviving shock from the green streptococcus protein were partly protected against the homologous P.

The nucleoproteins were also tested in guinea pigs sensitized with antibacterial sera. Since these sera contained antibodies for all the reactive substances but in different amounts depending on the method of immunization or the individual response of the rabbit, the tests with them were partly incidental to other experiments and were collected as Experiment 3.

Experiment 3.—Guinea pigs sensitized with 0.5 cc. to 1.0 cc. of antibacterial sera were tested 1 to 6 days later by intravenous injections of nucleoprotein P in doses ranging from 4 mg. to 10 mg. The M. A. D. was usually determined. The results are recorded in Table III.

The table is self-explanatory and shows that guinea pigs passively sensitized with antibacterial sera died in acute anaphylactic shock when injected intravenously with 7.5 mg. to 10 mg. of homologous or of heterologous hemolytic streptococcus nucleoprotein. Proteins from the different strains, except possibly from the homologous strain, were approximately equally effective in producing shock. Since homologous P solutions always contained some type-specific protein M, a smaller M.A.D. was to be expected. Precipitin tests, not tabulated here, also showed the presence of P antibodies in all these sera.

Active sensitization was obtained with nucleoprotein antigens in a few instances. After numerous doubtful results, unsuccessful apparently on account of dosage or timing between injections, the following satisfactory experiment in active anaphylaxis was performed.

Experiment 4.—Three guinea pigs were given intravenous injections of P from hemolytic or from green streptococci, as shown in Table IV. 22 days later each guinea pig was reinjected with 20 mg. of hemolytic streptococcus P. Table IV summarizes the experiment.

All three guinea pigs suffered typical anaphylactic shock on reinjection with hemolytic streptococcus proteins. Although the intensity

of the shock varied with different sensitizing antigens and was most marked with the most distantly related P, still the postmortem findings

TABLE III.

Anti-P Reactions.

Effect of Nucleoprotein, P, on Guinea Pigs Sensitized with Antibacterial Sera.

Guinea pig	Sensitized with serum*		Shocked by intravenous injections			
	No.	Cc.	Days after sensitization	Antigen: P from *S. hæmolyticus* strain	Dose	Result
					mg.	
C-1	R323	1.0	6	S39 (het.)	7.5	+
C-2	"	1.0	6	" "	10.0	++++
C-3	"	1.0	6	S60 (hom.)	10.0	†4 min.
C-4	R321	0.5	3	S3 (het.)	10.0	†4 "
C-5**	R261	0.5	1	" "	4.0	–
K-1	"	0.5	1	" "	10.0	†11 min.
C-6	"	1.0	6	S60 "	5.0	++
C-7	"	1.0	6	" "	7.5	†3.5 min.
C-8	"	1.0	6	" "	7.5	†45 "
C-9	"	1.0	6	" "	10.0	†3.5 "
M-2	Q308	1.0	3	S3 "	10.0	†3.5 "
M-3	"	1.0	2	" "	10.0	†50 "
M-6	"	1.0	3	" "	10.0	++
C-10	R446	0.5	1	S43 "	5.0	++
C-11	"	0.5	1	" "	7.5	+++
C-12	"	0.5	1	" "	10.0	†3.5 min.

* Serum R323 was against Strain S43, *Type* S60.
" R321 " " " " " "
" R261 " " " S39, " S23.
" Q308 " " " S23, " "
" R446 " " " S3, " S3.

** Three other guinea pigs in this group received the same dose with the same result.

were typical of anaphylactic death in all instances. This experiment offered sufficient evidence that nucleoprotein antigens from streptococci could sensitize guinea pigs actively.

These experiments confirm the conclusions previously reached as a result of precipitin tests and absorption experiments that the nucleoprotein fraction P is common to hemolytic streptococci and that it is related to similar protein fractions of green streptococci and of pneumococci.

The anaphylactic reactions of the second non-type-specific substance were next investigated. This is referred to as the C substance and is probably a complex carbohydrate chemically similar to the type-specific soluble substances of certain other bacteria. It will be recalled however, that this fraction, while it is species-specific for hemolytic

TABLE IV.

Anti-P Reactions.

Active Sensitization with Nucleoprotein Antigens.

Guinea pig	Sensitizing injection*			Shocking injection* 22 days later			
	Antigen P from strain**	Dose	Result	Weight	Antigen P from strain	Dose	Result
		mg.		*gm.*		*mg.*	
D-1	S43 (*S. hæmolyticus*)	20	−	135	S43 (*S. hæmolyticus*)	20	++++ (†overnight)***
D-2	S3 " "	20	±	186	" " "	20	†50 min.
D-3	V92 (" *viridans*)	20	−	173	" " "	20	†4 "

* Intravenous.

** Strain S43 represents *Type* S60; Strain S3 represents *Type* S3.

*** Lungs distended as in acute anaphylactic death.

streptococci, shows no type specificity whatever in its precipitin reactions. Thus, with antibacterial serum from Rabbit R446 typical disc precipitates were formed in high dilutions of C preparations from all strains of hemolytic streptococcus tested. Consequently, it was important to find out whether this antigen-antibody system could cause anaphylaxis, and the following experiment was performed to determine this point.

Experiment 5.—A series of guinea pigs was sensitized with Serum R446. On the following day the animals were tested with intravenous injections of varying amounts of "purified" C from two heterologous strains of hemolytic streptococcus. The results are given in Table V.

In this experiment typical anaphylactic shock was produced with a substance which is probably a carbohydrate. Titration of C from Strain S43 showed that 0.04 mg. was the M.A.D. for guinea pigs sensitized on the preceding day with Serum R446. Similarly, the M.A.D. of C from Strain S23 was 0.03 mg. The results exactly parallel the precipitin tests recorded in Table I of the preceding paper (3). A

TABLE V.

Anti-C Reactions.

Effect of Two Relatively Pure C Substances on Guinea Pigs Sensitized with Antibacterial Serum with a High Titer of C Precipitins. Determination of Minimal Anaphylactic Dose.

Guinea pigs sensitized with serum from a rabbit immunized with Strain S3, *Type* S3.

Guinea pig	Shocked by intravenous injections 1 day after sensitization		
	"Purified" C from strain	Dose	Result
		mg.	
E-1	S43 (het.)	0.02	++++
E-2	" "	0.04	†3.5 min.
E-3	" "	0.2	†3.5 "
E-4	" "	2.0	†4 "
E-5	S23 "	0.01	±
E-6	" "	0.03	†4 min.
E-7	" "	0.1	†5 "

The three strains used in this experiment represent three distinct types of *S. hæmolyticus:*

S3 represents *Type* S3.
S43 " " S60.
S23 " " S23.

crude C extracted with antiformin from another strain of *Type* S23 also produced typical anaphylactic death in guinea pigs sensitized with this serum. Precipitin tests with this extract were negative for M and for P substances, and paralleled the anaphylaxis results in being positive for the C substance. The results with this extract are not included in the table.

Since the C substance produced undoubted anaphylactic shock, the question of its chemical nature became of even greater interest and

theoretical importance than before. As already pointed out, it was supposed that this fraction was probably a carbohydrate, and the facts supporting this supposition have been considered previously (3), as well as the fact that the chemical analyses do not exclude the possibility that the active material is combined with protein or is protein itself. Exposure of C to active trypsin, however, did not alter its activity in the precipitin test, and the effect of such digestion on the anaphylactic reaction is shown in the following experiment.

TABLE VI.

Anti-C Reactions.

Exposure of C Substance to Trypsin: Effect on Anaphylactic Shock.

Guinea pigs sensitized with Serum R446 from a rabbit immunized with Strain S3, *Type* S3.

Guinea pig	Shocked by intravenous injections 1 day after sensitization		
	Purified C from Strain S23, *Type* S23, treated with:	Dose*	Result
		mg.	
F-1	Inactive trypsin	0.05	+++
F-2	" "	0.075	†3 min.
F-3	Active "	0.05	+++
F-4	" "	0.075	†3.5 min.

* These figures may be somewhat inaccurate on account of the small volumes with which the experiment was performed.

Experiment 6.—"Purified" C substance was subjected to the action of 0.5 per cent trypsin for 50 minutes. The technique of the experiment was the same as that employed in similar experiments described in previous papers (3). The trypsin was shown to be active by a simultaneous experiment in which the type-specific protein M was completely digested under the same conditions. (See Experiment 3 in the following paper.) Titrations of the C substance exposed to inactivated trypsin (the control) and of that exposed to active trypsin were made in guinea pigs sensitized with Serum R446.

Table VI shows that trypsin did not digest the C substance or change its titer in the anaphylactic reaction under conditions which completely destroyed other reactive fractions of the hemolytic streptococcus.

A few additional experiments were made to test the reactivity of C in guinea pigs sensitized with an anti-P serum and with antibacterial sera which showed only a low titer of C antibodies in the precipitin test. Experiment 7 gives some of these results.

Experiment 7.—Two guinea pigs, sensitized 2 days earlier with an anti-P serum, were given intravenous injections of C in doses which represented 3.3 and 20 M. A. D. respectively for animals sensitized with serum potent in C antibody.

TABLE VII.

Anti-C Reactions.

Effect of C Substances on Guinea Pigs Sensitized with:

(1) Anti-P Sera with No C Precipitins.

(2) Antibacterial Sera with Low Titer of C Precipitins.

Guinea pig	Sensitized with serum	Kind of serum*	Shocked by intravenous injections				
			Days after sensitization	Purified C extract from strain	Dose		Result
					Mg.	M.A.D.**	
G-1	R500	Anti-P	2	S23 (hom.)	0.1	3.3	—
G-2	"	"	2	" "	0.6	20.0	—***
G-3	R322	Antibacterial	1	S43 "	0.2	5.0	—***
G-4	R264	"	1	" (het.)	0.2	5.0	—
G-5	"	"	1	S23 (hom.)	0.1	3.3	—***

* R500 was a rabbit immunized with P from Strain S39, *Type* S23.
R322 was a rabbit immunized with Strain S43, *Type* S60.
R264 " " " " " " S39, " S23.

** M. A. D. in terms of Serum R446. See Table V.

*** Intravenous injections of suitable homologous P or M extracts on the following day produced typical anaphylactic death in these animals.

(See Experiment 5.) Since they were not shocked, the sensitiveness of one of these animals, G-2, was tested on the following day by an intravenous injection of nucleoprotein P.

Guinea pigs sensitized with antibacterial sera which had a low titer of C precipitins were also tested with C substances but showed no symptoms of shock, whether the C was obtained from homologous or from heterologous strains. Two of these animals, G-3 and G-5, subsequently received homologous M extracts to prove that they had been really sensitized.

This experiment emphasized the fact that this non-type-specific substance was not the same as the non-type-specific nucleoprotein P since it did not shock Guinea Pigs G-1 and G-2, sensitized with a potent anti-P serum. It also showed that C would shock guinea pigs sensitized with antibacterial sera, only if the serum had a relatively high content of C precipitins. Thus, G-3, G-4, and G-5 showed no shock when given 3 to 5 M.A.D. of C, although subsequent injections of homologous type-specific M extracts proved that this group of animals had been properly sensitized. These results parallel the precipitin reactions. This experiment will be referred to in another connection to show that the C substance is different from another non-type-specific substance (designated Y) which gave cross-reactions with certain antibacterial sera (notably, with Serum R264).

No attempts to sensitize actively with C could be made on account of the small amounts of material available.

DISCUSSION.

The anaphylactic reactions of two of the cell derivatives of *Streptococcus hæmolyticus* paralleled in every instance the precipitin reactions previously reported (3). The similarity of nucleoproteins (P) from different types as well as from different strains of hemolytic streptococci was again evident; while the partial relationships of P from related species of bacteria, such as *Streptococcus viridans* and pneumococcus, were also apparent. These conclusions are based on passive anaphylactic experiments with anti-P and with antibacterial sera and on active sensitization with P which was also accomplished.

The species-specific C substance was also effective in producing anaphylactic death in passively sensitized guinea pigs, but only if the serum showed a high titer of the non-type-specific C precipitins. Amounts as small as 0.03 mg. to 0.04 mg. were sufficient to cause death in such animals. This is an instance in which a substance, probably carbohydrate in nature, causes passive anaphylactic shock, an interesting finding in view of its chemical nature and the failure to immunize with it. It seems to be a hapten capable of reacting with antibody but not capable of stimulating antibody formation. Insufficient quantities have been available to determine its capacity to produce active anaphylaxis. A similar result has been reported since the conclusion of these experiments by Tomcsik (6) who obtained from *B. lactis*

aerogenes 5 gm. of a specific substance which was largely carbohydrate, although he could not free it of 0.9 per cent nitrogen. 0.03 mg. of this product was the minimal anaphylactic dose for guinea pigs passively sensitized with antibacterial serum, a figure strikingly similar to that obtained with the probable carbohydrate of the hemolytic streptococcus. Tomcsik's material was non-antigenic in that it did not produce active anaphylaxis in guinea pigs nor antibodies in rabbits into which it was injected. His work is analogous to that reported here with the C substance; and in both instances the final proof that the material is pure carbohydrate is still lacking, although the amount of nitrogen present in one minimal anaphylactic dose is certainly small. In the case of the streptococcus C, which contained approximately 4 per cent nitrogen, the minimal anaphylactic dose, therefore, contained only about 0.000001 gm. of nitrogen. In the present anaphylactic experiments, additional evidence that C is not a protein is the failure to digest it with trypsin; and its failure to shock guinea pigs passively sensitized with anti-P serum is further evidence that it is distinct from the non-type-specific nucleoprotein, P.

SUMMARY.

The anaphylactic reactions of two non-type-specific fractions of hemolytic streptococcus extracts parallel the precipitin reactions. The nucleoprotein, P, is a true antigen, in that it stimulates antibody production in rabbits, as shown before, and produces anaphylactic shock in guinea pigs actively as well as passively. The probable carbohydrate, C, on the other hand, does not induce antibody formation in rabbits, so far as known at present, but does produce typical anaphylactic shock in guinea pigs passively sensitized with antibacterial serum provided the serum shows a high titer of C precipitins. This is an instance of a hapten, probably carbohydrate in nature, causing anaphylactic shock in passively sensitized guinea pigs.

BIBLIOGRAPHY.

1. Zinsser, H., and Parker, J. T., *J. Exp, Med.*, 1917, xxvi, 411.
2. Dale, H. H., *J. Pharmacol. and Exp. Therap.*, 1912–13, iv, 167, 517.
3. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 397; 1928, xlvii, 469, 481.
4. Weil, R., *J. Med. Research*, 1913–14, xxiv, N. S., 233.
5. Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1913, xii, 341.
6. Tomcsik, J., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 812.

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

V. ANAPHYLAXIS WITH THE TYPE-SPECIFIC SUBSTANCE.

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The anaphylactic reactions caused by the group-reactive nucleoprotein P, and by the species-specific substance, C, obtained from extracts of hemolytic streptococcus have been shown in the preceding paper to parallel their precipitin reactions (1). Thus equivalent amounts of nucleoproteins derived from different types of hemolytic streptococci caused acute anaphylactic death in guinea pigs passively sensitized with anti-P serum prepared with nucleoprotein from any of these types. Moreover, nucleoproteins from related species of bacteria, *Streptococcus viridans* and pneumococcus, also caused anaphylactic death if injected into similarly sensitized guinea pigs in sufficiently large doses. This partial relationship had already been observed in precipitation and absorption experiments (2). The nucleoproteins were also capable of sensitizing guinea pigs in active anaphylactic experiments. The other non-type-specific fraction, the species-specific C substance, which seemed to be non-protein and probably carbohydrate, was also effective in producing acute anaphylactic death in passively sensitized guinea pigs. Only those antibacterial sera which showed a high titer of C antibodies in the precipitin test, however, were under the conditions of the experiments capable of sensitizing guinea pigs passively to shock with the C substance. As in the precipitin reaction, so also in the anaphylactic reaction, the C substance showed no type specificity but gave cross-reactions with sera prepared against all types of hemolytic streptococci. While the chemical data were not sufficient to prove conclusively that this substance was a carbohydrate, still they indicated that this was the case. They were substantiated by the failure of trypsin or of pepsin to affect

either the precipitin titer or the minimal anaphylactic dose of C. These two non-type-specific substances extracted from hemolytic streptococci differed both chemically and in their failure to cause reciprocal precipitin or anaphylactic reactions with their respective antisera.

The present experiments are concerned with the anaphylactic reactions of the type-specific protein, M, and with certain cross-reactions observed with some of the extracts containing M which may be due to a third non-type-specific fraction, Y.

Methods.

The preparation of extracts and of antisera has been described, and the technique of the anaphylactic test is the same as that employed with the non-type-specific fractions (1).

The protein fraction, M, which gave type-specific precipitin reactions, was tested for type-specific anaphylactic reactions as shown in Experiment 1.

Experiment 1.—Guinea pigs, sensitized with antibacterial serum, were tested with intravenous injections of homologous and of heterologous HCl extracts which contained the type-specific M. Surviving guinea pigs were reinjected on the following day with homologous M. The M.A.D. for each extract was determined, and doses were recorded as approximate multiples of the M.A.D. with a definite homologous serum. Control unsensitized guinea pigs were never shocked by these extracts. Table I shows some of the type-specific reactions.

Guinea pigs sensitized with eight different antibacterial sera showed only slight reactions when tested with intravenous injections of 2 to 50 M.A.D. of heterologous M, although the controls, similarly sensitized, died in acute anaphylactic shock when given 1 or 2 M.A.D. of homologous M. The animals surviving heterologous M, either succumbed to injection of homologous M on the following day or suffered a severe but sublethal shock. The increase in amount of homologous M required to produce death following shock with heterologous M was probably due to the presence in these extracts of traces of non-type-specific substances as impurities. Such an experiment was evidence of type-specific anaphylactic shock with the M fraction. A micro Kjeldahl nitrogen determination made on the most active lot

of HCl extract from Strain S43 showed that the M.A.D. for guinea pigs passively sensitized with homologous Serum R322 was 0.2 mg. of protein.

Active sensitization with HCl extracts was attempted; but, although doses and intervals between injections were varied considerably, no positive results were obtained. In view of the difficulty of finding the right conditions for sensitization with P, however, these negative results were not considered conclusive.

Occasional sera were encountered which gave non-type-specific anaphylactic, as well as precipitin, reactions with relatively purified HCl extracts from Strain S43. Since it was obvious that the type-specific M was not completely isolated but that traces of the other reactive fractions were almost certainly present, it was possible that these results were due to reactions of the latter with their respective antibodies which are usually present in antibacterial sera. Experiment 2 was performed to test this hypothesis.

Experiment 2.—Five guinea pigs, I-1 to I-5, sensitized with Type S23 antibacterial sera received intravenous injections of HCl extract from a strain of heterologous type, as shown in Table II. All these animals died in typical anaphylactic shock. In order to determine whether this cross-reaction was due to traces of the common nucleoprotein P, Guinea Pigs I-6 to I-12 were sensitized with a highly potent anti-P serum and were given intravenous injections of the extract used in the first part of the experiment. Such slight symptoms resulted that P was eliminated as the cause of the cross-reactions in I-1 to I-5. The extract was next tested for the presence of the other known commonly reactive substance, C, by injecting it into guinea pigs sensitized with an antibacterial serum of high potency for C antibody. Since none of these animals showed more than very slight symptoms, the common C substance was also eliminated as the cause of the cross-reactions.

All surviving guinea pigs (I-6 to I-16) were tested again, usually on the following day, with suitable homologous extracts. Typical anaphylactic death resulted, except in the cases of I-6 and I-7 which suffered + + + and + + shock respectively, thus showing that the animals had been effectively sensitized.

Table II shows the cross-reactions of occasional *Type* S23 antibacterial sera with certain HCl extracts from another type. The possible explanation that traces of the common substances, P and C, were responsible for these non-type-specific reactions was eliminated in each instance by testing the extract for the presence of P and of C

TABLE I.

Anti-M Reactions.

Type-Specific Anaphylactic Shock with Antibacterial Sera and HCl Extracts.

Guinea pig	Sensitized with serum*		Shocked by intravenous injections				
			Test No.	Days after sensitization	HCl extract from strain**	Dose M.A.D.‡	Result
	No.	Cc.					
H-1	R323	1.0	1	6	S43 (hom.)	20.0	†4.5 min.
H-2	"	1.0	1	6	S39 (het.)	2.0	±
			2	7	S43 (hom.)	1.0	†4 min.
H-3	R321	0.5	1	1	S43 (hom.)	1.0	†4.5 min.
H-4	"§	0.5	1	1	S39 (het.)	5.0	++
			2	3	" "	10.0	±
			3	4	S43 (hom.)	2.0	†4 min.
H-5	Q866	0.5	1	2	S43 (hom.)	2.0	†4 min.
H-6	"	0.5	1	2	S39 (het.)	2.5	±
			2	3	S43 (hom.)	2.0	++
H-7	Q864	0.5	1	2	S43 (hom.)	2.0	†3 min.
H-8	"	0.5	1	2	S39 (het.)	2.5	±
			2	3	S43 (hom.)	4.0	++
H-9	R322	0.5	1	2	S43 (hom.)	2.0	†4.5 min.
H-10	"	0.5	1	2	S39 (het.)	2.5	±?
			2	3	S43 (hom.)	6.0	+++
H-11	R324	0.5	1	2	S43 (hom.)	2.0	†4 min.
H-12	"	0.5	1	2	S39 (het.)	2.5	±?
			2	3	S43 (hom.)	6.0	†4 min.
H-13	Q613	1.0	1	1	S39 (hom.)	1.0	†7 min.
H-14	"	1.0	1	1	S43 (het.)	50.0	++
			2	3	S39 (hom.)	2.0	†3.5 min.
H-15	Q317	1.0	1	2	S39 (hom.)	1.0	++++
			2	3	" "	2.5	+++
H-16	"	1.0	1	2	S43 (het.)	2.0	±
			2	3	S39 (hom.)	2.5	+++

by injecting it into guinea pigs sensitized with sera of known high potency for P and for C antibodies. It was shown previously in the reciprocal experiment[1] that "purified" C did not shock guinea pigs sensitized with one of the sera which gave cross-reactions in the present experiment (Serum R264 used for I-5). This is further evidence that C is not the cause of the cross-reactions in the present instance. Since this hypothesis proved, therefore, untenable, it was necessary to assume either (1) that still another non-type-specific substance was present in the antigenic complex of the hemolytic streptococcus, or else (2) that the M substance was not strictly type-specific but that it might be nearly enough related to M from a different type to cross occasionally with an antibody which is *chiefly* specific for the latter. Considerable evidence has been accumulated indicating that the second hypothesis is incorrect. A comparison of the M.A.D. of different lots of extracts in terms of animals sensitized with homologous type serum and of others sensitized with heterologous type

[1] See Paper IV of this series, Table VII, G-4 and G-5.

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* Sera R321, R322, R323, and R324 were against Strain S43, *Type* S60.
" Q864 and Q866 " " " S60, " "
" Q613 " Q317 " " " S23, " S23.

** Strain S43 represents *Type* S60.
" S39 " " S23.

‡ M.A.D. of S43 extract in terms of Serum R321.
" " S39 " " " " " R261 or R264.

§ 6 other guinea pigs sensitized with this serum and tested with varying combinations of these 2 antigens reacted in the same way.

In all tables the following symbols are employed:

−	indicates	no shock.
±?	"	trace of shock.
±	"	slight shock.
+	"	mild shock.
++	"	moderate shock.
+++	"	moderately severe shock.
++++	"	severe shock.
+++++	"	very severe shock.
†	"	animal died.

hom. = homologous.
het. = heterologous.
M.A.D. = minimal anaphylactic dose.

TABLE II.

Anti-Y Reactions.

Non-type-Specific Anaphylactic Shock with Certain Antibacterial Sera and HCl Extracts.

Guinea pig	Sensitized with serum No.*	Shocked by intravenous injections of HCl extract from Strain S43, *Type* S60			
		Days after sensitization	M.A.D. in terms of serum against		Result
			homologous *Type* S60	heterologous *Type* S23	
Antibacterial sera giving cross-reactions with HCl extracts from a strain of another type					
I-1	Q308	2	2	2	†4 min.
I-2	"	4	20	1.3	†4 "
I-3	R261	6	2	2	†4.5 "
I-4	"	1	2	2	†3 "
I-5	R264	2	2	2	†6 "
Pure Anti-P sera					
I-6	R500	6	2.5	2.5	+
I-7	"	3	10	10	±?
I-8	"	1	10	10	+
I-9	"	1	10	10	+
I-10	"	1	2	2	+
I-11	R594	1	2	2	±?
I-12	"	3	10	10	−
Antibacterial serum containing C antibody					
I-13	R446	1	1.3	1	+
I-14	"	2	15	1	+?
I-15	"	1	1	1−	±
I-16	"	2	?**	3	±

* Guinea Pigs I-1, -2, -3, -5, -6, -11, -12 were sensitized with 1 cc. of serum; all others with 0.5 cc.

R500 was an anti-P serum against P from Strain S39, *Type* S23.

R594 " " " " " " " " S43, " S60.

All others were antibacterial sera:

R446 was against Strain S3, *Type* S3

Q308 " " " S23, " S23.

R261 " " " S39, " "

R264 " " " " " "

** HCl extract from Strain S39, *Type* S23, given to this animal.

sera shows wide variation. For example, with the extract used to test Guinea Pig I-1, the M.A.D. in terms of *Type* S23 and *Type* S60 sera was the same, while with an extract from the same strain prepared at another time (used for I-2) the M.A.D. in terms of heterologous *Type* S23 serum was fifteen times the M.A.D. in terms of homologous *Type* S60 serum. This difference in degree of heterologous activity of different lots of extract seems significant in indicating that M itself is not responsible for the cross-reaction, but rather that some non-type-specific substance, also extracted at times by HCl, is the cause of these atypical reactions.

The following experiment shows the effect of tryptic digestion on the anaphylactic reactions of this so called Y substance as well as its effect on the type-specific M.

Experiment 3.—A concentrated HCl extract from Strain S43 was digested with 0.5 per cent trypsin for 50 minutes with the technique previously described.[2] Controls with heated trypsin were included. Guinea Pig O-1, sensitized with serum prepared against Strain S43, served as control and died in acute anaphylactic shock on injection of 1 M.A.D. of the undigested extract (containing heated trypsin). Guinea Pig O-2, on the contrary, sensitized in the same way, was unaffected by the same dose of digested extract, although it was proved sensitive by injection on the next day of the same amount of untreated extract which resulted in typical anaphylactic death.

The same solution, known to give cross-reactions characteristic of the non-type-specific Y, was tested in Guinea Pigs O-3 and O-4, sensitized with serum prepared against a heterologous type strain. The control, O-3, died of anaphylactic shock when given 1 M.A.D. of the undigested extract, while O-4 suffered no shock from the same amount of digested extract. On the following day, however, O-4 was killed by 1 M.A.D. of untreated extract, thus proving that it was sensitive. Table III shows these results.

The results of this experiment were clear. Tryptic digestion destroyed the type-specific M contained in S43 HCl extract, as shown by the failure of the digested material to shock O-2, which had been sensitized with the homologous serum. The animal was sensitive, for it was killed by subsequent injection with untreated extract. The control animal, O-1, which received the undigested (heated trypsin) extract, died in 3 minutes. Similar results were obtained when the

[2] Experiment 3 described in Paper II of this series.

digested and the undigested S43 HCl extracts were tested in guinea pigs sensitized with heterologous Serum R264: the digested extract did not affect O-4 (although subsequent injection with untreated extract caused acute anaphylactic death), while the undigested control extract was still effective in shocking Guinea Pig O-3. Like all

TABLE III.

Tryptic Digestion of HCl Extracts Containing M and Y; Effect on Anaphylactic Shock.

Guinea pig	Sensitized with serum No.*	Shocked by intravenous injections of 1 to 1.5 M.A.D.					
		Test No.	Days after sensitization	HCl extract		Result	Probable chief cause of reaction
				from strain:	treated with:		
To show tryptic digestion of M							
O-1	R322	1	1	S43 (hom.)	Inactive trypsin	†3 min.	M
O-2	"	1	1	" "	Active trypsin	—	—
		2	2	" "	—	†4 min.	M
To show tryptic digestion of Y							
O-3	R264	1	1	S43 (het.)	Inactive trypsin	†3.5 min.	Y
O-4	"	1	1	" "	Active trypsin	—	—
		2	2	" "	—	†14 min.	Y

* Guinea pigs sensitized with 0.5 cc. of serum.

Serum R322 was an antibacterial serum from a rabbit immunized with Strain S43, *Type* S60; Serum R264 was an antibacterial serum from a rabbit immunized with Strain S39, *Type* S23

the other anaphylactic tests, this corroborated the previous experiments, the results of which were tested only by the precipitin reaction, and was further evidence that these two fractions were proteins. It did not, however, serve to show that this extract really contained two substances. The following absorption experiment differentiated them in such a way as to remove the doubt as to the existence of Y.

Experiment 4.—Three guinea pigs, sensitized with untreated serum from Rabbit R261, were used as controls on the activity of the extracts: J-1 died when tested with HCl extract from a heterologous strain, while J-2 and J-3 also died when tested with HCl extract from the homologous strain. Three other guinea pigs, J-4 to J-6, were sensitized with the same serum which had been absorbed by the technique previously described (2) with a strain (S24) known to be heterol-

TABLE IV.

Antibacterial Serum, R261, Rendered Type-Specific in Its Anaphylactic Reactions by Absorption with Heterologous Bacteria, Strain S24.

Guinea pig	Sensitized with Serum R261*		Shocked by intravenous injections				
	Cc.	Preliminary treatment of serum	Test No.	Days after sensitization	HCl extract from strain	Dose M.A.D.**	Result
J-1	0.5	None (control)	1	1	S43 (het.)	2	†3 min.
J-2	0.5	" " ‡	1	1	S39 (hom.)	1−?	†60 "
J-3	0.5	" " §	1	1	" "	1	†5 "
J-4	0.9	Absorbed	1	1	S39 (hom.)	1−?	+++
J-5	0.9	"	1	1	S43 (het.)	2	−
			2	2	S39 (hom.)	2−	†3.5 min.
J-6	0.45	"	1	1	S43 (het.)	10	−
			2	2	S39 (hom.)	2+	†5.5 hrs.

* Serum R261 was from a rabbit immunized with Strain S39, *Type* S23.

** All M.A.D. in terms of Serum R261.

‡ J-2 controls J-4 and J-5.

§ J-3 " J-6.

ogous to all the other strains used in this experiment. J-4, tested with the homologous HCl extract, suffered severe shock but recovered since too small a test dose was used; J-5 and J-6 were first tested with the same heterologous extract which had killed J-1, but they were unaffected by it. The next day, however, they succumbed to homologous HCl extract, thus showing that they had been sensitized. All necessary controls were negative. Table IV summarizes the results.

The serum used in this experiment sensitized animals to shock with heterologous HCl extracts, but after it was absorbed with entirely

heterologous bacteria, it induced only type-specific anaphylactic reactions. Guinea Pigs J-5 and J-6, sensitized with absorbed serum, withstood respectively 2 and 10 M.A.D. of heterologous HCl extract. Subsequent injection on the following day with the homologous extract resulted in acute anaphylactic death in the case of J-5 and in delayed death in the case of J-6, which, however, had been sensitized with only half as much absorbed serum as the other animals. The absorption method, therefore, eliminated the confusing cross-reactions and brought out type-specific anaphylactic as well as precipitin reactions, thus showing that M and Y must be different substances.

Tables V to VIII, inclusive, show how much was accomplished towards demonstrating type-specific anaphylactic reactions in this serum and in others which crossed with heterologous HCl extracts, by the method of desensitizing guinea pigs with extracts of various kinds and subsequently testing with the homologous extract containing M. This *in vivo* saturation of antibodies was comparable, in part, to the *in vitro* absorption described above. The results are somewhat complicated in this instance, however, by the necessity of using extracts which, although they contained one reactive substance in excess, contained in addition traces of others as impurities. Table V shows desensitization with one of these extracts.

Experiment 5.—Guinea Pigs K-1, K-2, and K-3 were sensitized with antibacterial serum, R261, the same serum used in absorption experiment, No. 4. On the following day at hourly intervals, five subcutaneous injections each of 10 mg. of NaOH extract (chiefly P) from heterologous Strain S3 were given to K-2 and K-3. 1 hour after the last injection, 20 mg. of this heterologous extract, given to K-2 intravenously, caused no symptoms, although 10 mg., given to the sensitized but otherwise untreated control K-1, caused typical anaphylactic death. At the same time, 20 mg. of the homologous NaOH extract (containing M as well as P) caused immediate death when given intravenously to the treated K-3.

This simple experiment showed that a sensitized guinea pig (K-2) was desensitized by subcutaneous injections of P so that it no longer reacted to twice the amount of P required to kill a similarly sensitized animal (K-1) which had not received these subcutaneous injections. Guinea Pig K-3, desensitized in the same way as K-2, was, however, killed by 20 mg. of homologous NaOH extract. Since this always contained the type-specific M, in addition to P, the experiment showed

that desensitization to P was accomplished by subcutaneous injections of P without desensitizing the animal to homologous M.

The effect of such desensitization with NaOH extracts on subsequent shock with HCl extracts was tested in Experiment 6.

TABLE V.

Antibacterial Serum, R261, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with Heterologous NaOH Extract on Subsequent Shock with Heterologous and with Homologous NaOH Extract.

Guinea pigs sensitized with 0.5 cc. of serum, R261, from a rabbit immunized with Strain S39, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections 1 day after sensitization				
		NaOH extract from strain	Dose	Result	Probable chief cause of reaction	Remarks
			mg.			
K-1	None (control)	S3 (het.)*	10**	†11 min.	P	
K-2	5 subcutaneous injections totaling 50 mg. (5 M.A.D.) of NaOH extract from heterologous strain, S3, 1 day after sensitization	" "	20	±?‡	—	Desensitized to P
K-3	Same as K-2	S39 (hom.)	20	†4 min.	M	Not desensitized to hom. M

* Strain S3 represents *Type* S3.

** 1 M.A.D.

‡ A subsequent test on this animal with homologous M (HCl extract), following a test immaterial to this experiment, resulted in severe shock.

Experiment 6.—Guinea Pigs L-1 to L-4 were sensitized with another Type S23 serum which induced cross-anaphylactic reactions with HCl extracts from a heterologous type. L-1 and L-2 were controls on the activity of the extracts: L-1 succumbed to an intravenous injection of heterologous HCl extract, and L-2 to homologous HCl extract. 2 days after sensitization at hourly intervals L-3 and L-4 were given subcutaneous and intraperitoneal injections of NaOH ex-

tracts from a heterologous strain increasing in dosage from 5 mg. to 16.5 mg. until a total of 50 mg. had been administered. The following day the heterologous HCl

TABLE VI.

Antibacterial Serum, R264, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with NaOH Extract, from a Strain Heterologous to the Serum and to All Test Extracts, on Subsequent Shock with Heterologous and with Homologous HCl Extracts.

Guinea pigs sensitized with 0.5 cc. of serum, R264, from a rabbit immunized with Strain S39, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	HCl extract from strain*	Dose M.A.D. in terms of serum No.** R 264	Dose M.A.D. in terms of serum No.** R 321	Result	Probable chief cause of reaction	Remarks
L-1	None (control)	1	2	S43 (het.)	2	2	†6 min.	Y	
L-2	" "	1	1	S39 (hom.)	1	0	†5 "	M	
L-3	5 subcutaneous and intraperitoneal injections, totaling 50 mg. (5 M.A.D.) of NaOH extract from heterologous Strain S3, 2 days after sensitization	1	3	S43 (het.)	2	2	—	—‡	L-3 and L-4 show a certain amount of desensitization to Y but not to hom. M
L-4	Same as L-3	1	3	" "	4	4	+++	Y	
		2	4	S39 (hom.)	5	0	†3.5 min.	M	

* Strain S3 represents *Type* S3.
" S43 " " S60.
" S39 " " S23.

** Serum R264 was against Strain S39, *Type* S23.
" R321 " " " S43, " S60.

‡ Found dead next morning; blood culture sterile.

extract was titrated by giving the desensitized guinea pigs injections of 2 and 4 M.A.D. of this extract. The smaller dose produced no shock in L-3, and the larger dose produced moderately severe, but not fatal, shock in L-4. The latter

animal was killed by 5 M.A.D. of homologous HCl extract given intravenously the next day.

This experiment showed that NaOH extracts contained substances which could desensitize guinea pigs to a certain extent to shock with heterologous HCl extract containing the non-type-specific Y. It seems probable that this is due to the presence of the Y substance in both extracts. A similar experiment was performed with another *Type* S23 serum, as shown in Table VII.

Experiment 7.—This experiment differed from Experiment 6 in the use of *Type* S23 serum prepared against another strain. The first four animals sensitized with this serum served as controls on the activity of the extracts. M-5 was desensitized by subcutaneous and intraperitoneal injections of NaOH extract from an entirely heterologous strain by the same method as L-3 and L-4 in the last experiment. The following day it survived an injection of more than 2 M.A.D. of heterologous HCl extract but was killed on the next day by the homologous HCl extract. M-6 was desensitized by one intravenous injection of 10 mg. of heterologous NaOH extract, a usually fatal dose, which did not kill this animal. The following day it showed very slight symptoms on injection of heterologous HCl extract but died the next day after an injection of homologous HCl extract. See Table VII for details of the experiment.

In this experiment, which is similar to Experiment 6, desensitization with entirely heterologous NaOH extract given subcutaneously and intraperitoneally or intravenously, resulted in some degree of desensitization with respect to the heterologous HCl extract which previously had killed animals sensitized with this serum. These animals, however, were not desensitized to the homologous HCl extract, for both were killed by intravenous injections of homologous HCl extracts, which in the case of M-6 was in a dose comparable in size to the dose of heterologous HCl extract used on the preceding day. Here again the desensitization was probably due to Y contained in both NaOH and HCl extracts.

In the next experiment the animals were desensitized by preliminary injections of heterologous HCl extract instead of NaOH extract and the effect of desensitization tested for both these extracts as well as for the homologous HCl extract.

Experiment 8.—A series of guinea pigs was sensitized with 1 cc. of the same serum used in the last experiment. Two of these animals served as controls on

TABLE VII.

Antibacterial Serum, Q308, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with NaOH Extract, from a Strain Heterologous to the Serum and to All Test Extracts, on Subsequent Shock with Heterologous and with Homologous HCl Extracts.

Guinea pigs sensitized with 1 cc. of serum, Q308, from a rabbit immunized with Strain S23, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	Extract from strain*	Kind of extract	Dose M.A.D. in terms of serum No.** Q308	Dose M.A.D. in terms of serum No.** R322	Result	Probable chief cause of reaction
M-1	None (control)	1	2	S43 (het.)	HCl	1	7.5	†4 min.	Y
M-2	" "	1	3	S3 "	NaOH	1±‡	1	†3.5 "	P
M-3	" "	1	2	" "	"	1±	1	†50 "	"
M-4	" "	1	1	S39 (hom.)	HCl	1	0	†4 "	M
M-5	5 subcutaneous and intraperitoneal injections, totaling 150 mg. (15 M.A.D.), of NaOH extract from heterologous Strain S3, 1 day after sensitization	1	2	S43 (het.)	"	2.7	20	—	—
		2	3	S39 (hom.)	"	10	0	†3.5 min.	M
M-6	None (desensitized by intravenous injections)	1	3	S3 (het.)	NaOH	1±‡	1	++	P
		2	4	S43 "	HCl	1.3	10	±	Y
		3	5	S39 (hom.)	"	1.5	0	†30 min.	M

* Strain S3 represents *Type* S3.
" S43 " " S60.
" S39 " " S23.

** Serum Q308 was against Strain S23, *Type* S23.
" R322 " " " S43, " S60.

‡ 10 mg.

the effectiveness of the antigens; N-1 was killed by an intravenous injection of 2 M.A.D. of heterologous S43 HCl extract, and M-4 by 1 M.A.D. of homologous S39 HCl extract.

Guinea Pig N-3 was desensitized 2 days after sensitization by subcutaneous injections of 15 M.A.D. (in terms of both homologous and of heterologous sera) of heterologous S43 HCl extract. The next day this animal gave practically no reaction to an intravenous injection of 4 M.A.D. of the same extract, while it

TABLE VIII.

Antibacterial Serum, Q308, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with Heterologous HCl Extract on Subsequent Shock with Heterologous NaOH Extract and with Heterologous and Homologous HCl Extracts.

Guinea pigs sensitized with 1 cc. of serum, Q308, from a rabbit immunized with Strain S23, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	Extract from strain*	Kind of extract	Dose* M.A.D. in terms of serum No. Q308	Dose* M.A.D. in terms of serum No. R322	Result	Probable chief cause of reaction
N-1	None (control)	1	2	S43 (het.)	HCl	2	2	†4 min.	Y
M-4	" "	1	1	S39 (hom.)	"	1	0	†4 "	M
N-3	3 subcutaneous injections, totaling 15 M.A.D. of S43 HCl extract 2 days after sensitization	1	3	S43 (het.)	"	4	4	±	Y
		2	4	S39 (hom.)	"	5	0	†4 min.	M
N-4	None (desensitized by intravenous injections)	1	2	S43 (het.)	"	0.7	5	++	Y
		2	4	" "	"	4.7	35	—	—
		3	5	S3 "	NaOH	2±	2**	—	—
		4	6	S39 (hom.)	HCl	5	0	†22 min.	M

* See foot-notes * and **, Table VII.

** 20 mg. See Guinea Pigs M-2, M-3, and M-6, Table VII, for controls on the M.A.D. of this extract.

died in 4 minutes on the following day after an intravenous injection of 5 M.A.D. of homologous S39 HCl extract.

Guinea Pig N-4 was desensitized by intravenous injections of heterologous S43 HCl extract. 2 days after sensitization, 0.7 M.A.D. of this solution caused ++ shock; 2 days later 4.7 M.A.D. of the same solution produced no effect; and the next day, 2 M.A.D. of NaOH extract from an entirely different strain, S3, caused

no symptoms. On the following day, however, the animal died a typical anaphylactic death, though delayed to 22 minutes, following an intravenous injection of 5 M.A.D. of homologous S39 HCl extract. Table VIII gives these results.

This experiment was the reciprocal of Experiments 6 and 7. In the latter, heterologous NaOH extract was used to desensitize against HCl extract from a heterologous strain of still another type. In Experiment 8 the reverse procedure was employed: the heterologous HCl extract was used to desensitize against the NaOH extract. In both instances, a certain degree of reciprocal desensitization was obtained without as much desensitization for the homologous HCl extract. These sera, with which it had previously been impossible to demonstrate type-specific anaphylactic reactions, were, therefore, rendered type-specific in that the desensitized animals reacted slightly, or not at all, with several multiples of the M.A.D. of heterologous extracts and were killed by similar, or even smaller doses, of homologous extracts.

If no other evidence were available, the cross-desensitization between NaOH extracts and HCl extracts could be explained as due to the presence in both solutions of the non-type-specific substances, C and P, but it has already been shown in Experiment 2 that the cross-reactions of the HCl extracts cannot be due to C or to P. Since the desensitization does not prevent the reaction with the homologous extracts, it seems improbable that it could be accounted for as non-specific reduction of reactivity such as is occasioned by injection of peptones or other foreign proteins. The correct explanation, therefore, seems to be the assumption of this other non-type-specific substance, Y, in extracts of the hemolytic streptococcus.

DISCUSSION.

Type-specific anaphylactic reactions were easily obtained with the protein, M, (HCl extract) when injected into guinea pigs passively sensitized with most of the antibacterial sera used in these experiments. Four sera produced by immunizing rabbits with strains of one type were, however, encountered, which caused non-type-specific anaphylactic death in passively sensitized guinea pigs when HCl extract from a strain of another type was used for the intoxicating injection. In order to obtain type-specific anaphylactic shock in guinea pigs pas-

sively sensitized with these sera, it was necessary either (1) to absorb the serum with heterologous bacteria or (2) to desensitize the passively sensitized guinea pigs with heterologous extracts. Since the HCl extract responsible for these atypical reactions did not cause reactions in guinea pigs sensitized with potent anti-P sera or with sera potent in C antibody and, conversely, since the C substance did not cause reactions with these atypical antibacterial sera, it was necessary to assume that another non-type-specific substance was present in HCl extracts and in the hemolytic streptococcus itself. The additional fact that this so called Y substance was digested by trypsin, while C was not, eliminated the possibility that C and Y were the same substance. Reciprocal desensitization experiments with heterologous NaOH extracts and with the HCl extract which gave the atypical crossing, showed some cross-desensitization for these heterologous extracts though not for homologous, a fact probably explainable as due to the presence of varying mixtures of all the non-type-specific substances in these extracts.

Active sensitization was not accomplished with M although numerous attempts were made under varying conditions, a result which agrees with another negative result, namely, the failure so far to immunize rabbits with this substance. In both instances, however, the failure must be taken with reserve, since it is possible that some change in technique might reverse the result. Active sensitization with Y (as contained in HCl extracts) was not observed.

A certain amount of additional information as to the nature and relationships of some of the reactive substances contained in the hemolytic streptococcus has been obtained by the use of the anaphylactic reaction. It is certain that the type-specific M produced typical anaphylactic shock in passively sensitized guinea pigs. *A priori*, this fact might be considered an indication that the M substance could function as an antigen in inducing antibody production: actually, all immunization experiments with rabbits and active sensitization experiments with guinea pigs yielded negative results. But in view of the additional evidence that C, which seems to be carbohydrate in nature, also shocked passively sensitized guinea pigs and never gave evidence of antibody stimulation, it seems probably that these two substances are haptens which react with antibodies produced by the

intact bacteria, when tested by means of the precipitin reaction and the passive anaphylactic reaction, but that they have no power to elicit antibodies themselves.

The facility with which acute anaphylactic death was produced by injecting bacterial extracts into passively sensitized guinea pigs was striking. Several factors are probably involved. Many investigators have found it necessary to resort to the Dale method with the excised uterus on account of the primary toxicity of the extracts to be tested. Zinsser and Parker (3) found this true in working with extracts of typhoid bacilli, and later Zinsser and Mallory (4) had the same experience with pneumococcus extracts and even with that method found that the margin between the dose which caused reactions in the normal uterus and that which caused it in the sensitive uterus was not so great as in anaphylaxis with egg albumin or with horse serum. With the hemolytic streptococcus, however, such difficulties fortunately were not encountered, and normal guinea pigs did not react to many multiples of the test doses. The fact that relatively large amounts of concentrated extracts were available and that the serological activity, as well as the number and kind of substances present, was capable of titration in most instances contributed to the success of these experiments. An analysis of the antibodies present in the immune sera used for passive sensitization was also made by means of the precipitin test, with the result that sera with known antibody content were used in the anaphylactic experiments and the control of the results was, therefore, increased.

SUMMARY.

1. Type-specific anaphylactic shock was produced with HCl extracts of *Streptococcus hæmolyticus* in guinea pigs passively sensitized with antibacterial sera.

2. With occasional sera and certain HCl extracts, type-specific shock was not produced unless the serum was first absorbed with heterologous bacteria or unless the guinea pigs were desensitized with heterologous extracts before testing with the homologous extract. The findings indicated that this was due to the presence of the non-type-specific substance which has been provisionally designated as Y.

3. Tryptic digestion destroyed the ability of HCl extracts containing

the type-specific M substance and the non-type-specific Y substance to produce anaphylactic shock in passively sensitized guinea pigs.

4. Active sensitization was not accomplished with the type-specific M. It seems probable, therefore, that this substance is a hapten, reacting with antibodies but not stimulating their production after separation from the bacterial cell.

BIBLIOGRAPHY.

1. Lancefield, R. C., *J. Exp. Med.*, 1928, xlvii, 843.
2. Lancefield, R. C., *J. Exp. Med.*, 1928, xlvii, 91.
3. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1917, xxvi, 411.
4. Zinsser, H., and Mallory, T. B., *J. Immunol.*, 1924, ix, 75.

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BACTERIAL VARIATION IN CULTURES OF FRIEDLÄNDER'S BACILLUS.

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PLATES 36 AND 37.

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During the course of studies on the biological and immunological properties of Friedländer's bacillus, at least three sharply defined types were found (1) to exist among different strains of the organism. The types were designated A, B, C, and into one Group, X, were placed several heterogeneous strains. That Friedländer's bacillus, moreover, possesses at least two different cellular constituents both of which are important in the antigenicity of the organism was recorded in later communications (2, 3). One of these is now known to be polysaccharide and is chemically different for each of the fixed types (4, 5). The differential specificity of the types appears to depend on the polysaccharides, a class of substances which are analogous to the soluble specific substance of Pneumococcus, originally described by Dochez and Avery (6). The second substance is protein in nature and regardless of type derivation exhibits the undifferentiated characteristics of the species. To this extent, Friedländer's bacillus possesses an antigenic complex which is analogous to that of Pneumococcus (7) as already described in papers from this laboratory.

It has been pointed out by other investigators (8–14) as well as ourselves that there are certain conditions which induce in Friedländer's bacillus the development of variants, a phenomenon which is now considered as bacterial dissociation.[1] The typical colonies of Friedländer's bacillus are now identified as smooth (S) and the variant colonies as rough (R), the organisms in each instance differing not only morphologically, but biologically and antigenically. The most salient

[1] For an analytic and critical review of the literature on microbic dissociation, the reader is referred to Hadley's monograph in the *J. Infect. Dis.*, 1927, xl, 1.

differences are (2) that the S cells form colonies with a smooth surface; they are virulent, encapsulated, and type-specific; and they produce the soluble specific substance. On the other hand, the R cells form colonies with a rough surface; they are avirulent, capsule-free, and not type-specific, but species-specific; and they do not elaborate the soluble specific substance. Furthermore, sera prepared by immunization with S cells are type-specific and passively protect against fatal infection by bacilli of the same type, while anti-R sera are not type-specific and possess no demonstrable protective properties for infected mice.

Further study has since disclosed that there exist among the R variants of Friedländer's bacillus additional dissociates which present definite differences in morphology and antigenicity. Moreover, observations have been made upon some of the conditions conducive to variation, the possible reversibility of the variants to their antecedent forms, and the occurrence of variants in human infection. The present communication comprises a report of these studies.

That several morphological forms or intermediates exist between the extremes of the R and S varieties of the Friedländer's bacillus has been reported by Toenniessen, Baerthlein, and Hadley. The work of the two former writers antedated the use of the present nomenclature.

EXPERIMENTAL.

Without entering into a discussion as to what constitutes a Friedländer's bacillus, the S strains studied in this investigation were all members of the *Encapsulatus* group as determined by cultural and staining reactions. They were all Gram-negative, encapsulated bacilli, which were virulent for mice and showed great variation in sugar fermentation reactions. The strain "SC" (Type A) which was most studied in the present investigation, was isolated from the blood of a fatal case of pneumonia in man. It fermented without gas, dextrose, sucrose, maltose, mannitol, and lactose.

The R strains employed in the previous study were derived by consecutive transfer of S cells in broth to which was added a concentration of 10 per cent homologous immune serum. Plate cultures were made after each transplant until R colonies were grossly visible. In the earlier studies, the R colonies were not examined for structural differences, but at a later period, however, three different forms of R colonies were recognized.

The characteristic colonies of the S and R variety have been observed with each of the serological types of Friedländer's bacillus. A detailed

study, however, has been made of those derived from Type A only, and the data, unless otherwise stated, refer solely to this type.

I. Forms of Variants Encountered.

In addition to the S variety, three different forms of colonies have been recognized. For the sake of convenience and clarity of expression these three R variants may be designated as R1, R2, and R3. Further than gross appearance the R3 colony was not studied, because it was found only rarely and sporadically in mass cultures of R cells and particularly because it was extremely unstable so that the organisms were never obtained in pure culture. The R3 colony was of the "phantom" description, escaping notice when viewed by transmitted light on account of its marked transparency. By reflected light, with most of the light obscured, it was seen as a transparent colony with a smooth surface and an annular margin which appeared slightly raised and fringed.

(*a*) *Morphological Differences.*—The S colony, examined by transmitted light after 15–18 hours of growth, appears opaque, white, sharply circumscribed, markedly convex, homogeneous, and circular (see Fig. 1). When seeded heavily the colonies coalesce, and this mucoid coalescent growth is typically characteristic of S organisms. By reflected light the surface of the S colony is glistening and smooth and reflects sharply and accurately the image of objects within focal distance. The colony growth is mucoid and tenacious in consistency, and is elastic to the touch of a needle. The S organism is Gram-negative, and encapsulated (Figs. 2 and 3). The rods are short and thick and most commonly occur in either single or in diplo forms.

The R1 colony by transmitted light appears transparent, pale yellow or tan in color with a slightly indented border; it is flat with a distinct central papilla that becomes more prominent with age; it is not homogeneous, and it is more or less circular in shape (see Fig. 1). By reflected light, the surface appears uneven and glossy and reflects images definitely but in a distorted fashion. Due to the manner in which the light is reflected the center of the colony seems to be raised into a small cone. The colony growth is more discrete, never as tenacious as S, and is readily picked with a needle. Stained preparations exhibits a short, slender, almost coccoid bacillus, which is Gram-

negative and unencapsulated. The forms are often so small as to suggest morphologically *B. influenzæ* (Figs. 4 and 5).

The R2 colony by transmitted light appears transparent, pale yellow or tan in color, with wavy margins; it is flat, not homogeneous, but appears matted simulating a tuft of cotton (see Fig. 1). The shape of the colony varies, but in general it is circular. By transmitted light, the surface markings suggest an oyster shell, *i.e.* irregular concentric rings with rough surface, and the reflection of images is always distorted. The colony growth is not confluent, mucoid, or tenacious and is picked readily. Stained preparations show long, slender, Gram-negative bacilli which, in young cultures, often occur in long wavy chains or threads. The organism is not encapsulated (Figs. 6 and 7).

The size of the S and the R colonies varies considerably, but in a general way, the R2 colonies are the largest, the S next in size, and the R1 are the smallest. Morphologically R2 bacillus is the longest while the R1 is the shortest. The S, on the other hand, shows the greatest dimension in breadth.

Figs. 1 to 7 demonstrate the relative differences of S, R1, and R2, both in colony formation and in microscopical appearance.

(*b*) *Biological Differences.*—Culturally, the S and R forms exhibit as striking differences as they do morphologically. The growth of the S cells in fluid media is viscous and accompanied by the production of soluble specific substance. The S form is always encapsulated and of marked virulence for mice, the intraperitoneal injection of 1/10 millionth cc. of a young culture (6–8 hours) causing death within 24–48 hours. In such instances, the peritoneal exudate is viscous, contains relatively few leucocytes which are frequently surrounded by a clear zone separating them from the bacilli, and phagocytosis of the organism has never been observed in normal animals.

The growth of R1 and R2 in fluid media is diffuse, non-viscous, and not accompanied by the elaboration of specific soluble substance. The organisms are not encapsulated and the virulence of both forms is extremely low, since doses as large as 0.5 cc. of a young culture frequently fail to cause fatal infection in mice. The peritoneal exudate following the injection of R1 or R2 is not viscous and contains numerous leucocytes which are able apparently to phagocyte the bacteria.

The fermentation reactions of the three strains were also studied.

The carbohydrates tested were dextrose, lactose, sucrose, maltose, and mannitol. As will be seen in Table I, both the S and the R strains fermented each of the sugars within the first 24 hours of growth, except that in the case of R1, the fermentation of lactose on the two occasions tested did not occur until the 6th day.

(*c*) *Antigenic Differences.*—It has already been shown in a previous communication (3) that S strains of Friedländer's bacillus induce in rabbits the formation of antibodies which agglutinate type-specifically, precipitate the corresponding specific soluble substance, and protect white mice against infection by strains of the same type. Anti-S sera may in addition contain species-specific antibody depending upon

TABLE I.

Biological Reactions of S, R1, and R2 Strains of Friedländer's Bacillus (Type A).*

Strain	Capsule	Specific soluble substance	Virulence	Phagocytosis	Fermentation reactions				
					Lactose	Dextrose	Sucrose	Mannitol	Maltose
S	+	+	+	−	+	+	+	+	+
R1	−	−	−	+	+x	+	+	+	+
R2	−	−	−	+	+	+	+	+	+

* The S strain was isolated from the blood of a fatal case of pneumonia in man.

+ indicates presence.

− indicates absence.

x, fermentation was delayed to 6th day, whereas all the other fermentations occurred within 24 hours.

the duration and intensity of the immunization. Consequently, anti-S sera may cause agglutination of R cells. Anti-R sera, on the other hand, are lacking in antibodies associated with type specificity and protection, and contain only the common group antibody which reacts with R organisms derived from any of the serological types. The R strains employed in these reactions, however, were mass R cultures and represented one colony arising from the continued growth of an S strain in homologous anti-S serum.

The antigenic character of R1 and R2 was correlated with that of the original S strain from which they had arisen. Antisera were prepared by the intravenous injection of rabbits with heat-killed suspensions (2) of S, R1, and R2, respectively. With the resulting immune

sera, it was established that S was agglutinated in anti-S sera, but not in either R1, or R2, antisera, as is brought out in Table II. R1 and R2 were agglutinated in anti-S serum to a slight extent, the reaction appearing granular in contradistinction to the disc reaction occurring with S in anti-S sera (2).

TABLE II.

Cross-Agglutination Reactions with S, R1, and R2 Strains of Friedländer's Bacillus (Type A).

Strain	Immune serum					
	Anti-S		Anti-R1		Anti-R2	
	1:5	1:10	1:5	1:10	1:5	1:10
S	++++	++++	−	−	−	−
R1	++	+	++++	++++	++++	++++
R2	++	+	++++	++++	++++	++++

In this and following tables ++++ indicates complete agglutination with flocculent precipitate and clear supernatant; +++, almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; −, no agglutination.

TABLE III.

Cross-Agglutination Reactions with R1 and R2 Strains of Friedländer's Bacillus (Type A).

Immune serum	Strain	Final dilution of serum									
		5	10	20	40	80	160	320	640	1280	2560
Anti-R1	R1	++++	++++	++++	++++	++++	+++	+++	++	+	−
	R2	++++	++++	++++	++++	+++	+++	++	+	−	−
Anti-R2	R1	++++	++++	++++	++++	+++	+++	++	++	+	+
	R2	++++	++++	++++	++++	++++	++++	+++	+++	++	+

The immune serum of R1 was found to agglutinate both R1 and R2 to about the same extent, and conversely R2 antiserum caused an equally good agglutination of both strains. Agglutination was the typical R variety of granular sedimentation which breaks up readily upon agitation. The antigenic similarity evidenced by the agglutina-

tion reaction was further studied by means of agglutinin adsorption. R1 and R2 immune sera were adsorbed with both strains, and then tested for the presence or absence of agglutinins. The results of the experiments can be summarized briefly: Each strain (R1 and R2) adsorbs from the homologous antiserum agglutinins for both homologous and heterologous organisms; from the heterologous serum, however, antibody is removed only for the strain employed in the adsorption. In other words, R1 and R2 possess mutual agglutinating characters, but not complete, mutual adsorptive properties, as deter-

TABLE IV.

Agglutinin Adsorption Reaction.

Results of Agglutination with R1 and R2 Serum after Adsorption with R1 and R2 Strains.

Immune serum	Adsorbed with	Antigen	Final dilution of serum							
			20	40	80	160	320	640	1280	2560
Anti-R1	R1	R1	−	−	−	−	−	−	−	−
		R2	−	−	−	−	−	−	−	−
	R2	R1	++++	++++	++++	+++	++	++	+	−
		R2	−	−	−	−	−	−	−	−
Anti-R2	R1	R1	−	−	−	−	−	−	−	−
		R2	++++	++++	++++	++++	+++	+++	++	+
	R2	R1	−	−	−	−	−	−	−	−
		R2	−	−	−	−	−	−	−	−

mined by reciprocal adsorption. The data of these experiments are presented in Table IV.

As was to be expected from previous results (2) neither R1 nor R2 immune sera caused agglutination of the S strain from which they originated, nor did they cause precipitation of specific soluble substance, nor passively confer immunity upon mice infected with the antecedent S strain.

II. The Reversibility of R to S.

The R strains studied in this investigation have been remarkably stable during the 2 years they have been under observation. Since

the permanency of R becomes of paramount importance when viewed in terms of the problems of infection and epidemiology, experiments were planned to determine the reversibility of R to S. The older literature particularly with other species offers some evidence in favor of reversibility, but the objection has been raised that mass cultures were studied instead of pure line strains. More recently, however, it has been shown unimpeachably that single cell cultures of R may be caused to revert to S under proper cultural conditions. Thus Jordan (15) and Soule (16) showed interconvertibility of *B. paratyphosus* B, Levinthal (17), and Dawson and Avery (18), of Pneumococcus, Soule (19), of *B. subtilis*.

In the present study of reversibility, single cell strains were obtained by the technique of Avery and Leland (20). Since all experiments with pure line strains uniformly failed to bring about reversion, mass R cultures were studied instead, because such cultures might contain individual organisms with greater potentialities for reversion than the single R cells chosen at random. The observations were made with cultures derived from each of the three serological types. The methods adopted for reversion were (1) rapid transfer through meat infusion broth, (2) rapid transfer through dextrose broth, (3) growth in the supernatant culture fluid of the parent S strains, (4) growth in anti-R sera, (5) passage through normal white mice both before and after preliminary transfer through anti-R sera. The greater part of the experiments were carried out before our recognition of the two distinct forms of R variants and the mass cultures studied may have been mixtures of both forms. The results obtained with each method are briefly summarized below.

(*1*) *Rapid Transfer through Meat Infusion Broth.*—One strain each of both mass and single cell R cultures, derived from the three serologically different types, was carried through 90 transfers in meat infusion broth. Transplants were made two or three times daily and from time to time plates were streaked to examine colony formation and the cultures were tested for agglutination by the homologous anti-S serum. The reversion of R to S was not observed by this method.

(*2*) *Rapid Transfer through Dextrose Broth.*—It had been noted earlier in the study that a number of R strains which fermented

dextrose, grew in this medium in conglomerate clumps or masses strongly suggestive of a thread reaction. It is interesting to note in this connection that this phenomenon was never noticed in acid fermentation by S strains. Two mass cultures of R forms derived from a single colony were transplanted once daily in 1 per cent dextrose broth for 35 transfers. By the sixth subculture, clumped growth no longer occurred, although dextrose was still fermented. No evidence, however, was obtained of reversion.

(*3*) *Growth in the Supernatant of the Parent S Strain.*—18 hour broth cultures of S strains were centrifuged and the supernatant was withdrawn and rendered sterile by heating at 56°C. for 30 minutes. This was added in 10 per cent concentration to infusion broth alone and to infusion broth containing 10 per cent anti-R serum. In such media mass R cultures were transplanted twice daily for 90 transfers. At no time during the period of observation was reversion encountered.

(*4*) *Growth in Anti-R Sera.*—Both mass and single cell R strains derived from each of the serological types were carried twice daily through 10 per cent anti-R serum broth for 90 generations. The anti-R serum used in the different experiments was both homologous and heterologous and later, mass cultures of R organisms derived from Type A were carried through 40 transplants in 1 per cent and 5 per cent anti-R serum broth. In the earlier transplants growth always appeared in thread formation, that is, clumped in the bottom of the tube with a clear supernatant fluid. After 20 to 40 or more transplants this reaction disappeared and growth was uniformly diffused. Although in some instances the colony growth seemed to be somewhat less rough, nevertheless, reversion did not occur.

(*5*) *Passage through Normal White Mice.*—Each mass culture from the preceding experiment (*i.e.* after 90 transplants in anti-R serum broth) was passed through normal mice by intraperitoneal injection. As controls, two other R strains—one derived from Type A and the other from Type B—were passed through mice without preliminary growth in anti-R sera. Mice were injected with large amounts of young R cultures and the peritoneal washings reinjected into other normal mice. This was carried out with each strain through a series of 22 mice but in no instance did reversion occur.

(*6*) *Experiment with R1 and R2.*—The foregoing experiments on the reversion of R to S were carried out as stated with either mass cultures or pure line strains without regard to the particular form of the R variant studied. It seemed possible, however, that the question might now be answered more accurately and completely by a study of the two well defined variant forms R1 and R2. Cultures of each variety, therefore, were transferred twice daily in broth to which had been added in one series 10 per cent homologous immune serum, and in another series 10 per cent heterologous immune serum. The strains were grown in this way for 60 transplants and after 30 to 38 transfers the thread reaction had disappeared. Under these conditions it was possible to induce R1 to change to R2 but reversion of either variant to the S type was not observed.

In summarizing, then, the results of the study of reversion, it may be stated that none of the methods employed, succeeded in bringing R forms back to the S type.

This does not mean, however, that R forms are irreversible, but that under the conditions stated, the methods employed were not adequate to effect the change.

III. Some Incitants to Variation.

(*a*) *Experimental Derivation of R Forms.*—Mass R cultures may be experimentally derived by the continued subculture of S cells in broth to which has been added homologous immune serum, R organisms gradually appear as the S forms disappear. It is an old observation among earlier workers, however, that Friedländer's bacillus upon aging gives rise to variant colonies which differ strikingly in certain characters, the authors reporting on some or all of the properties of virulence, agglutination, and colony appearance. Our experience corroborates these results and included the isolation of R forms from aged colonies on plates and occasionally from broth cultures stored for several weeks, in which the change has spontaneously occurred.

(*b*) *Occurrence of R Forms in Disease.*—It is definitely known that R variants may be experimentally derived *in vitro* from S cells. The phenomenon of bacterial dissociation, however, would acquire greater significance if it could be demonstrated that the process actually takes place in the animal body during the course of infection. In order to

study this possibility, a survey was made of strains freshly isolated from a number of different pathological conditions and a careful search was made for the presence of R variants. In all, cultures from seventeen different sources were examined and these included seven cases of human pneumonia, one case of pneumonia in a guinea pig, two of liver abscesses in man, two of acute and fatal abscesses in guinea pigs, two of cystitis in man, one of infected antrum in man, and two cases of infected adenoid tissue. In five instances R forms were iso-

TABLE V.

The Occurrence of R Variants in Infections Associated with Friedländer's Bacillus.

Case No.	Source	Type	Presence of R
1	Sputum, Autopsy } pneumonia	A	Not found
2	Sputum—pneumonia	A	Not found
3	Sputum—pneumonia	A	Not found
4	Sputum—pneumonia	A	Not found
5	Sputum—pneumonia	A	Not found
6	Abscess—guinea pig (fatal)	A	Not found
7	Abscess—guinea pig (fatal)	A	Not found
8	Liver abscess	A	Not found
9	Adenoid tissue	A	Present
10	Adenoid tissue	A	Present
11	Autopsy—pneumonia (guinea pig)	B	Not found
12	Sputum—pneumonia	B	Not found
13	Infected antrum	C	Present
14	Sputum—pneumonia	Group X	Not found
15	Urine—cystitis	Group X	Not found
16	Urine—cystitis	Group X	Present
17	Liver abscess	Group X	Present

Except where stated, the strains were derived from human infections.

lated and in each they were present in mixtures of R and S. Since the occurrence of the two distinct variants, R1 and R2, was recognized only after this survey was completed, it is impossible to state the relative frequency of these two forms. However, of the R strains isolated, two were present with S organisms of Type A, one with those of Type C, and two others in association with S cells of Group X. Interestingly enough, the R strains were found not in acute infections but in chronic conditions. Thus R forms were present twice in cultures from adenoid

tissue, twice in cases of chronic cystitis, and once from a subacute antrum. Suggestive as the data are, no generalization, however, can be made from so few observations. The details of this study are recorded in Table V.

DISCUSSION.

The study of variation in cultures of Friedländer's bacillus reported in the present communication discloses three different forms of R variants. Two of the variants (R1 and R2) have been studied in detail, and they may be recognized grossly by colony formation or microscopcally by the size and arrangement of the individual cells. Moreover, it has been possible to differentiate the dissociates further by serological reactions. Both variants (R1 and R2) are agglutinated in antisera prepared by injection of rabbits with either strain, but they lack the capacity of complete reciprocal agglutinin adsorption. The two R strains are markedly different from their antecedent S strain in colony appearance, morphology, virulence, and antigenicity.

A number of methods have been adopted to induce reversion of R to S. Whether the technique or its application was inadequate, the results were uniformly negative. This does not imply, however, that all R forms of Friedländer's bacillus are irreversible, but that in the case of the strains studied, the proper stimulus was not supplied by the methods used. In this connection the work of Dawson and Avery (21) offers an interesting comparison. They found one R strain of Type I pneumococcus irreversible by the identical methods which caused other R strains of the same and different types to change to the S form. In the present study, R2 has been converted to R1, while, on the other hand, R1 itself has remained unchanged following numerous transplants in homologous immune serum. Conversion of R2 to R1 and the less rough appearance of R1 colonies make it not unlikely that R1 is an intermediary form between S and R2.

The spontaneous development of R variants in S cultures of Friedländer's bacillus has been found to accompany the process of aging. Growth in immune sera *in vitro* also converts the S cells into R forms. That variation, however, is more than an *in vitro* or cultural degradation gains support from the fact that R forms have been found in cultures taken directly from foci of infection in the animal body caused

by Friedländer's bacillus. It is an interesting observation that in the cases studied R variants were found only in chronic infections and always in conjunction with S forms.

CONCLUSIONS.

1. Under proper conditions mass R cultures of Friedländer's bacillus may give rise to a number of variants which are dissimilar in colony appearance and morphology. Three such forms have been described. In two varieties, differences have been observed not only in colony formation and morphology, but also in cultural and antigenic characters.

2. None of the methods employed were adequate to cause reversion of any of the R variants to the S type. Growth of the R2 variant in its own antiserum, however, induced a change to the R1 form.

3. R forms of Friedländer's bacillus may be derived from S strains by aging or by growth in anti-S serum of the homologous type.

4. R strains may be isolated in culture directly from infection. In the cases where R forms were found, S cells were also present, and the pathological condition was of a chronic nature.

BIBLIOGRAPHY.

1. Julianelle, L. A., *J. Exp. Med.*, 1926, xliv, 113.
2. Julianelle, L. A., *J. Exp. Med.*, 1926, xliv, 683.
3. Julianelle, L. A., *J. Exp. Med.*, 1926, xliv, 735.
4. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 701, 709.
5. Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1927, xlvi, 601.
6. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 447.
7. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, xlii, 367; 1923, xxxviii, 73; 1924, xl, 301.
8. Streit, H., *Centr. Bakt., 1. Abt., Orig.*, 1906, xl, 709.
9. Beham, L. M., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxvi, 110.
10. Toenniessen, E., *Centr. Bakt., 1. Abt., Orig.*, 1914, lxxiii, 241.
11. Baerthlein, K., *Centr. Bakt., 1. Abt., Orig.*, 1918, lxxxi, 369.
12. Friel, A. R., *Pub. South. African Inst. Med. Research, No. 5*, 1915.
13. Small, J. C., and Julianelle, L. A., *J. Infect. Dis.*, 1923, xxxii, 456.
14. Hadley, P., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiii, 109; *J. Infect Dis.*, 1927, xl, 1
15. Jordan, E. O., *J. Am. Med. Assn.*, 1926, lxxxvi, 177.

16. Soule, M. H., *J. Bact.*, 1928, xv, 39.
17. Levinthal, W., *Klin. Woch.*, 1926, ii, 2020.
18. Dawson, M. H., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 943.
19. Soule, M. H., *J. Bact.*, 1927, xiii, 41.
20. Avery, R., and Leland, S. J., *J. Exp. Med.*, 1927, xlv, 1003.
21. Personal communication.

EXPLANATION OF PLATES.

PLATE 36.

FIG. 1. Plate culture of Type A, Friedländer's bacillus, taken by transmitted light. The S colony and the two variant forms, R1 and R2, are labelled. Note opacity of S and transparency of both R varieties.

FIG. 2. Smear of peritoneal exudate of mouse infected with S. Stained with Gram, × 1000. Note absence of leucocytes and presence of large capsules.

FIG. 3. Smear of S grown on agar. Gram stain, × 1000. Capsule is greatly diminished.

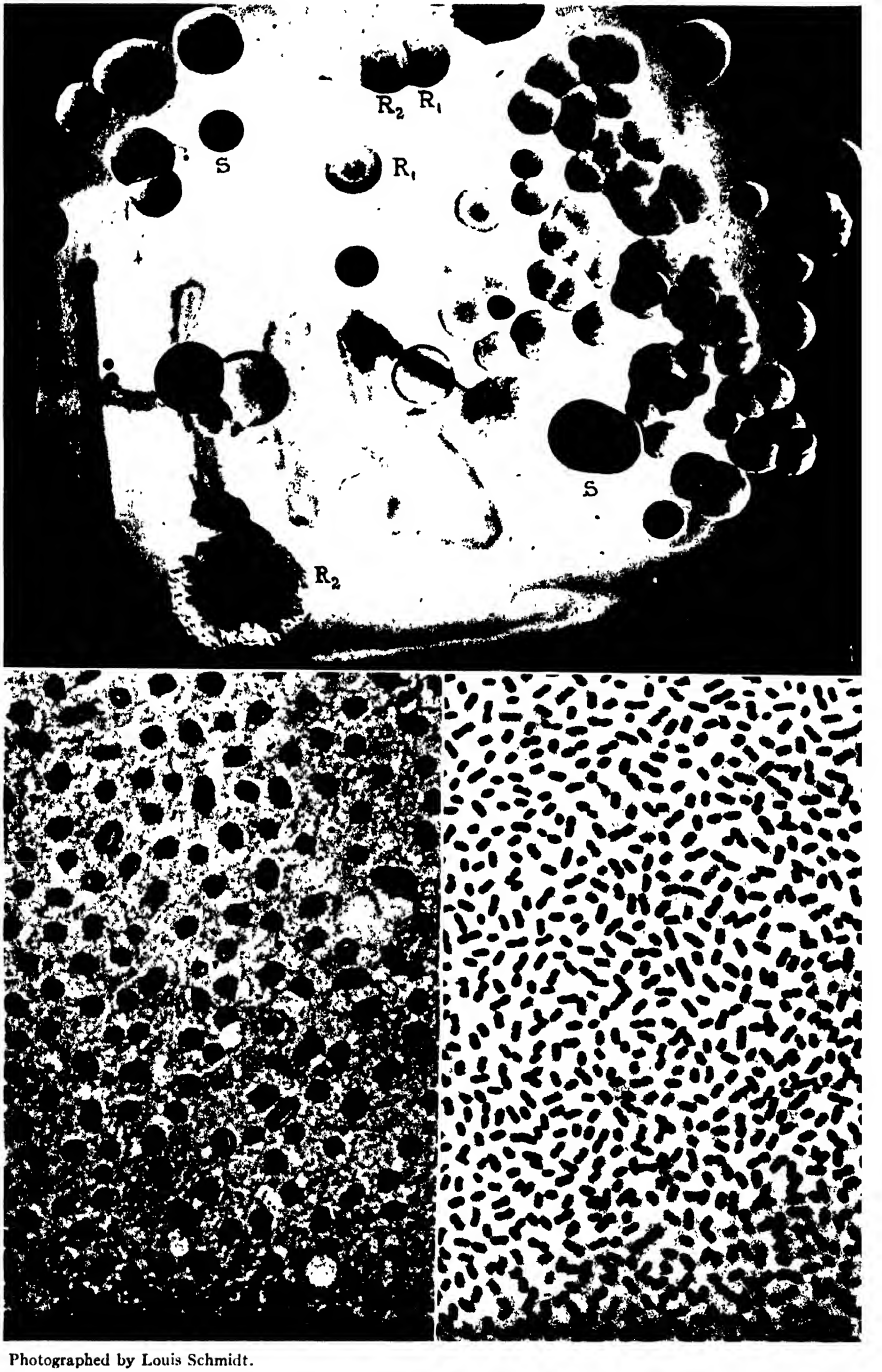
PLATE 37.

FIG. 4. Smear of peritoneal exudate of mouse injected with R1. Stained with Gram, × 1000. Note phagocytosis and lack of capsules.

FIG. 5. Smear of R1 grown on agar. Gram stain, × 1000. Note size and arrangement as contrasted with S and lack of capsules.

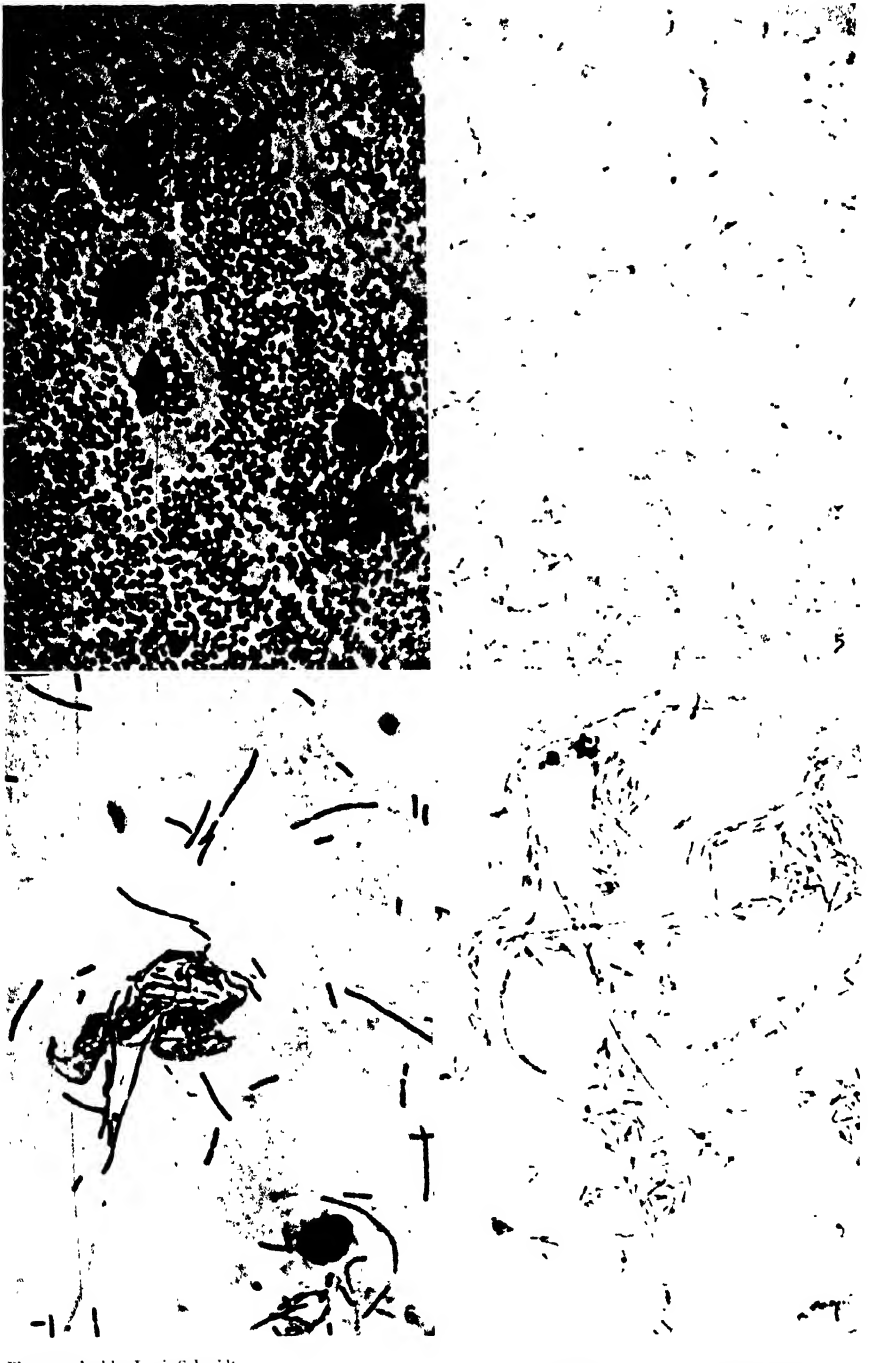
FIG. 6. Smear of peritoneal exudate of mouse injected with R2. Stained with Gram, × 1000. Note phagocytosis, lack of capsules, and length of rods.

FIG. 7 Smear of R2 grown on agar. Gram stain, × 1000. Note size and arrangement and lack of capsule.



Photographed by Louis Schmidt.

(Julianelle: Cultures of Friedländer's bacillus.)



Photographed by Louis Schmidt.

(Julianelle: Cultures of Friedländer's bacillus.)

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STUDIES ON THE POLYHEDRAL DISEASES OF INSECTS DUE TO FILTERABLE VIRUSES.

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INTRODUCTION.

The so-called polyhedral affections of insects are extremely interesting and economically important diseases, and much has been written upon the subject. They have been described in many different larvæ. However, cases described from forms other than those belonging to the Lepidoptera are questionable. These maladies are widespread among the moth larvæ of America, Europe, Asia, and probably elsewhere, and affect many of our worst pests, producing at times huge epidemics amongst them. Such epidemics, when they occur, contribute much more towards the control of certain of our noxious caterpillars than the combined efforts of all their hymenopterous and dipterous parasites. J. W. Chapman and the writer (1), in 1915 and 1916, listed twenty-two species, belonging to ten separate families, that are definitely known to be naturally susceptible to one of the polyhedral maladies. A useful form, the silkworm, is also unfortunately subject to one of these affections. The silk industry in certain regions suffers annually due to its ravages and no satisfactory practical remedy for it's control has yet been devised.

Paillot (2), in 1926, gave an excellent review of all the literature to date so the present article will concern itself primarily with those more recent works that have a direct bearing on certain questions still perplexing. The most important matters emphasized by J. W. Chapman and the writer (3) in their work on the wilt of gipsy moth and tent caterpillars, army worms and other species may be summarized:

1. The polyhedral diseases occur in many widely different species of lepidopterous larvæ.
2. They are highly infectious and often produce huge epidemics.
3. They are characterized by a rapid postmortem lysis of all the tissues.

4. They are identified by curious nuclear changes occurring within certain cells, especially within the hypodermal, tracheal matrix, fat and blood cells. These changes are accompanied by the intranuclear development of the so-called polyhedral bodies, which are later liberated into the blood on the disintegration of the cells.

5. Due to the fact that these well defined nuclear changes also occur in certain blood cells, the blood is a fairly reliable index of conditions in the other tissues, and can be used for diagnostic purposes as well as for a study of the progress of the disease.

6. The infectious agent is a filterable virus. This virus easily passes through Berkefeld "N" candles. It is difficult to pass through Berkefeld "W" candles and cannot be passed through Pasteur-Chamberland "F" filters.

7. Berkefeld filtrates are free from bacteria and polyhedral bodies.

8. The polyhedral bodies vary in size and shape in different species; they vary greatly in size within one species and even within a single individual.

9. Evidence, based on the filtration experiments, on staining reactions and on certain other chemical and physical tests, was advanced to show that the polyhedral bodies must be regarded as nucleoprotein reaction or by-products of the disease.

10. Infection naturally occurs through the mouth by means of the food, but the disease can also be experimentally reproduced through inoculation.

11. The incubation period varies, but is generally of longer duration than is the case with bacterial infections in insects, and temperature bears an important relation to this variation.

12. Certain individuals, although infected, do not acquire the disease—they are immune.

So many chronic carriers of wilt exist among our gipsy moths, that Chapman and the writer finally resorted to breeding and selection from healthy cultures to produce a disease-free race for experimental purposes. This method proved more satisfactory than others, as a comparison between our experimentally infected and control animals showed. However, gipsy moth wilt is so prevalent in New England that by using all conceivable precautions, spontaneous cases appeared in our stock and in our controls. The appearance of spontaneous cases among controls and the ruination of apparently well executed experiments is an old question, well known to European and American workers in this subject. Our results of that time were therefore subject to certain reserves of interpretation, the foregoing summary representing rather a set of tentatively formulated opinions than final conclusions.

Acqua (4) confirmed our filtration experiments in principle, using the grasserie or "jaundice" virus of silkworms instead of the gipsy moth wilt virus.

Aoki and Chigasaki (5) in some experiments with grasserie claimed that polyhedral bodies shaken in physiological salt solution, and centrifuged more than ten times were just as infectious for silkworms as the unwashed bodies. The entire work of these authors is devoted to proving that the polyhedra are parasitic.

Komarek and Breindl (6) in some work on the "Wipfelkrankheit" of nun moth larvæ came to the conclusion that the polyhedra are not the actual parasites, but that the etiological agent resides within them. These workers believe that the bodies are to be compared with galls and like them are produced as a specific reaction against the stimulation of the causative agent. The virus also exists free and as such is able to pass through Berkefeld filters.

Paillot (2, 7) found that grasserie silkworm blood, freed from cellular elements and polyhedra by centrifugation and filtration through three layers of filter paper was virulent. He also discovered that he could initiate grasserie by inoculating healthy worms with the blood of worms infected two days previously and in which no polyhedra could be detected at that time.

It thus appears that our conception that the disease is due to a virus separable from the polyhedral bodies and filterable through Berkefeld "N" filters has been amply confirmed. There still appear to be many unsolved problems in connection with this interesting group of diseases and work with them has been continued with the following questions particularly in mind.

1. Since the polyhedral diseases of insects are caused by filterable viruses, what is the essential nature of any particular virus; where is it localized in the body and can it be seen?
2. What is the nature of the polyhedral bodies?
3. Is a particular virus specific for a particular host or not? In other words, does more than one type of virus exist?
4. Do infected females sometimes survive, and do any of these transmit the virus to the next generation through their eggs?

To answer some of these questions tent caterpillars (*Malacosoma americana*) and silkworms (*Bombyx mori*) were employed. Tent

wilt occurs naturally in the region where these experiments were conducted, but it was not very prevalent during the seasons of 1923, 1924, 1925, and 1926. For this reason, it was not difficult to find healthy material for experimental purposes. Silkworm grasserie was obtained from France in 1924, through the kindness of Prof. A. Paillot. The writer had reared large numbers of silkworms for other studies during 1922 and 1923 without encountering a single case of grasserie. The first cases ever seen by him were those produced with Prof. Paillot's material. Therefore, it can be safely assumed that the stock, from which we drew our animals for the experiments, was entirely free from polyhedral infection. By the use of suitable precautionary measures, it has been possible to keep this stock free from spontaneous grasserie up to the present time.

Filtration Experiments.

Table I, page 511, represents infection experiments performed on tent caterpillars. The infectious material was derived from a single typical case of polyhedral disease found in the field. The tissues of the wilted larva were completely lysed and polyhedral bodies were abundant in the light brown liquid which oozed forth on rupturing the skin. This larva did not emit an offensive odor and stained films from some of the material did not reveal any bacteria. 0.5 cc. of this liquid from the dead larva was diluted with 5 cc. of sterile, distilled water and fed to four, 4th stage, healthy tent larvæ by smearing the material on apple leaves. The blood of these animals, prior to infection, was carefully examined for the presence of polyhedra in the nuclei of the blood cells, to eliminate the possibility of natural wilt. This precaution was taken with all the *Malacosoma* experiments in spite of the fact that only larvæ from disease-free nests were chosen.

As represented in Table I, Experiment 1, the four infected larvæ died of typical wilt in from 8–9 days. The body fluid from one of the dead larvæ was expressed and diluted in 50 cc. of sterile water. This material was passed through paper and then through a sterile Berkefeld "N" candle. The Berkefeld filtrate was tested on a variety of media for sterility and then fed to ten tent larvæ. Ten controls were held, fed with the same filtrate heated at 60°C. for 30 minutes. Experiment 2 records the results. Among those fed virulent filtrate,

TABLE I.

Tent Larvæ Experiments.

Exp. No.	No. of Tent Larvæ	Treatment	Larvæ Died of Wilt	Pupæ Died of Wilt	Died of Other Causes	Lived and Transformed to Moths	No. of days from Infection to Death
1	4	Fed emulsified tent wilt cadaver.	4				8–9
2	10	Fed Berkefeld filtrate.	8	1		1	7–17
	10	Fed Berkefeld filtrate heated 60°C. 30 minutes.				10	
3	20	Fed emulsified tent wilt cadaver.	14	5	1		4–10
	20	Fed emulsified tent wilt cadaver heated 60°C. 30 minutes.	1		2	17	
4	15	Fed Berkefeld filtrate.	11	1	3		7–10
	15	Fed Berkefeld filtrate heated 60°C. 30 minutes.			1	14	
5	10	Inoc. Berkefeld filtrate.	6		2	2	9–14
	10	Inoc. Berkefeld filtrate heated 60°C. 30 minutes.			3	7	
6	10	Fed Berkefeld filtrate centrifuged 4 hr. 2,000 R. P. M., top layer.	5	2		3	8–18
	10	Fed Berkefeld filtrate centrifuged 4 hr. 2,000 R. P. M., bottom layer.	5	1		4	8–16
7	10	Fed emulsified silkworm grasserie cadavers.			2	8	
8	10	Fed emulsified silkworm grasserie cadavers.				10	
9	10	Inoc. grasserie Berkefeld filtrate.			2	8	
10	10	Inoc. grasserie Berkefeld filtrate.			3	7	
11	10	Fed emulsified gypsy wilt cadavers.			3	7	
12	10	Inoc. gypsy wilt Berkefeld filtrate.			1	9	

8 died in the larval and 1 in the pupal stage in from 7–17 days. One lived and transformed into a moth. All of the controls lived and transformed into adults.

Experiments 3 and 4 constitute a continuation of the series, the infections produced with an individual dead of wilt in the preceding experiment. One control died of the disease in Experiment 3. This individual must have become infected accidentally during the course of the experiments. Deaths from "other causes" are also recorded in Experiments 3 and 4. These were all bacterial in nature. Experiment 5 represents infections produced by inoculating 0.1 cc. of the filtrate, prepared with physiological saline, under the skin.

Experiment 6 was performed to see if the virus could be concencentrated by centrifuging. A Berkefeld filtrate was centrifuged for 4 hours at 2,000 R. P. M., after which ten larvæ were fed with the top layer and ten with the bottom layer. No significant difference in virulence was observed. In all the experiments the minimum period from infection to death was 4 and the maximum 18 days. The average length of this period, about 10 days, is considerably more prolonged than is the case with the bacterial infections of insects. This fact was also recorded by Paillot (2) in 1926.

Experiments 1 to 5 inclusive, Table II, represent a series of infections in silkworms produced with Prof. Paillot's grasserie material. Experiment 1 was performed for the purpose of producing a quantity of material. A tube of grasserie blood, still containing cellular blood elements and polyhedra, was received in shipment from France. This blood was sterile for bacteria and was diluted five times with physiological saline solution and 0.1 cc. inoculated into each of 20 worms. After about 3 days a few of the worms began to show the characteristic jaundiced appearance (yellow discoloration of the white skin), and the first death occurred in 5 days.

Subsequently 11 more worms and 3 pupæ died typically of grasserie while 3 transformed into moths. Two died from the immediate effects of the inoculation. From one of these worms a Berkefeld "N" filtrate was produced as outlined in the experiments with tent wilt, and Experiment 2 performed. The series was continued to Experiment 5 inclusive. In the experiments dealing with infection by feeding, the virus was prepared with sterile, distilled water and

TABLE II.

Silkworm Experiments.

Exp. No.	No. of Silkworms	Treatment	Worms Died of Grasserie	Pupæ Died of Grasserie	Died of Other Causes	Lived and Transformed to Moths	No. of days from Infection to Death
1	20	Inoculated with grasserie blood.	12	3	2	3	5–16
2	20	Inoculated with Berkefeld filtrate.	14	1	1	4	6–20
	20	Inoculated with Berkefeld filtrate heated 60°C. 30 min.			3	17	
3	15	Inoculated with Berkefeld filtrate.	11	2		2	6–19
	15	Inoculated with Berkefeld filtrate heated 60°C. 30 min.			1	14	
4	15	Fed Berkefeld filtrate.	9	1		5	14–24
	15	Fed Berkefeld filtrate heated 60°C. 30 min.				15	
5	15	Fed Berkefeld filtrate.	12	2		1	12–20
	15	Fed Berkefeld filtrate heated 60°C. 30 min.				15	
6	10	Fed emulsified tent wilt cadavers.			2	8	
7	10	Fed emulsified tent wilt cadavers.			1	9	
8	10	Inoculated with tent wilt Berkefeld filtrate.			1	9	
9	10	Inoculated with tent wilt Berkefeld filtrate.			1	9	
10	10	Fed emulsified gypsy wilt cadavers.			3	7	
11	10	Inoculated with gypsy wilt Berkefeld filtrate.			1 (and 1 escaped)	8	

smeared on mulberry leaves; in the inoculation experiments sterile physiological saline solution was substituted, and 0.1 cc. inoculated subcutaneously.

By consulting Table II it will be seen that no controls died of grasserie; most of the grasserie deaths occurred in the larval, a few in the pupal stage. The "other cause" deaths were due to the direct effects of the inoculation. During the inoculation of such creatures as insect larvæ, it was often impossible to avoid puncturing the gut, although a small needle was used and great precaution exercised. Most of the bacterial deaths can be traced to this source. It is shown in the table that although the worms were either inoculated or fed virulent virus, a certain proportion resisted infection and transformed to adults. This transformation into adults, of a certain number of infected larvæ, was also noticed in the experiments with the tent caterpillar virus. In the grasserie experiments the minimum period from infection to death was 5 and the maximum 24 days. The average length of this period approximately corresponds to that given for the tent wilt experiments.

Portions of all Berkefeld filtrates, employed in the tent caterpillar and silkworm infection experiments, were plated out on various laboratory media and used only when these remained entirely sterile. Furthermore, portions of the filtrates were always centrifuged and the upper layer, as well as the bottom sediment, examined microscopically in the stained and unstained condition. No bacteria or polyhedral bodies were revealed, nor was anything else seen that one could interpret with certainty as having any etiological significance. Under the dark field illumination, with high magnifications, many minute, dancing granules were observed in the Berkefeld filtrates. Similar minute dancing granules, under similar circumstances, were also seen by the writer (3) in 1915 in his work on gipsy moth wilt. Apparently the same granules can often be seen free in the lymph of diseased caterpillars or worms and within the cytoplasm of pathological cells; especially within the nucleus during the earlier phases of the disease. These identical granules do not appear in the lymph nor within the cells of normal animals. Naturally it is difficult to decide what they are, but the writer is not impressed with their etiological significance and is inclined to believe that they constitute products of cellular disintegration.

The greatest number of filtration experiments were performed with the grade "N" Berkefeld filtrates. However, experiments were also undertaken with the grades "V" and "W" candles and with the Pasteur-Chamberland "F" filter. No lethal difference was noticed between the "V" and "N" filtrates. However, out of 20 silkworms and 20 tent larvæ inoculated with their respective viruses passed through "W" filters, only 4 tent larvæ and 1 silkworm died of disease. Twenty tent larvæ and 20 silkworms inoculated with filtrates of their respective viruses passed through Pasteur-Chamberland "F" filters, lived and transformed. All the filtrations were performed at a pressure of 74 mm.

From the experiments performed on tent caterpillars and silkworms, one is justified in concluding that the viruses of tent wilt and grasserie are easily filterable through Berkefeld "V" and "N" candles. They can be filtered through "W" candles only with difficulty, and cannot be passed through Pasteur-Chamberland filters. These results may depend upon the size or formation of the filter pores; upon the flexibility or rigidity of the virus particles; upon the electrical charge, or upon some other factor. The virulent filtrates are free from polyhedral bodies, bacteria, or any other formed body which can at present be made microscopically visible.*

Experiments with Washed and Fragmented Polyhedra.

J. W. Chapman and the writer (1, 3), in work on the wilt of gipsy moth larvæ, emphasized the fact that the polyhedral bodies are of great diagnostic value, but are not etiologically related to the disease. This opinion was based on positive results of certain filtration experiments which excluded the polyhedra, on the microscopical appearance of these bodies, their staining reactions, and on their physical and chemical nature. However, the work of Aoki and Chigasaki (5) and of Komarek and Breindl (6) again enveloped the above opinion in doubt. As previously mentioned, the two Japanese workers did much to attempt to prove that the polyhedra are parasitic. Komarek and

* The classification of filters is the one given in the Arthur H. Thomas Company, Philadelphia, 1926 catalog. *Berkefeld filters:* "W," fine; "N," medium; "V," coarse. *Pasteur-Chamberland filters:* "F," coarse texture and suitable for ordinary filtrations; "B," fine and suitable only for pressure.

Breindl claimed that the virus resides within these bodies. For this reason two further experiments were undertaken.

At separate times, two lots of polyhedral material were prepared; one was derived from a large number of tent wilt cadavers; the other from silkworm grasserie cadavers. All of the larvæ used had been dead only 3 days and on death were immediately placed on ice, to inhibit bacterial development. It is well known that the intestinal flora of herbivorous larvæ is extremely low unless a bacterial disease prevails. In each case, after enough material had accumulated, the dead larvæ were crushed in a mortar with physiological salt solution. This was then passed through cheesecloth, diluted a little more, and forced through a hard, fine pored paper (Whatman No. 50) by means of suction. The filtrate which abounded in cellular debris and polyhedra was then washed with physiological saline solution and centrifuged repeatedly, 20 times in the case of the tent and 22 times in the case of the silkworm material. This process extended over approximately 5 days, and during the interval between each cleansing process, the sediment was held on ice. After this, when it was thought that most of the virus had been removed, the final bottom sediment obtained had the gross appearance of white chalk, and on microscopic examination consisted almost entirely of polyhedral bodies, including a few urate and other crystals. The bottom sediment, which in each case amounted to about a gram of polyhedra, was shaken up with 5 cc. of physiological salt solution. 0.1 cc. of this milky white liquid was injected into each of 20 larvæ. Twenty tent larvæ were injected with the wilt polyhedra suspension, and 20 silkworms with the grasserie polyhedra suspension. Out of the entire 40 larvæ so infected, 7 died of a bacterial septicemia and 33 transformed into moths. The blood of all inoculated worms was examined twice during the course of the experiment, at an interval of one week, but polyhedra were never seen in the plasma nor within the nuclei of the cells.

The above experiment was repeated the following year with the exception that pains were taken to fragment most of the polyhedra by grinding in a mortar. This process was followed microscopically. Further fragmentation can be obtained by pressing them between two glass panes. Twenty tent larvæ and 20 silkworms were again injected

with their respective polyhedra. Thirteen died of bacterial septicemia and 27 transformed into moths. Blood examinations undertaken, as previously, were entirely negative.

It seems to us that the experiments described, when taken in conjunction with the filtration experiments, are an aid in eliminating the possibility that the polyhedra are parasitic or that they enclose a parasite.

THE HISTO- AND CYTOPATHOLOGY OF THE POLYHEDRAL DISEASES.

The Polyhedral Bodies, Their Origin and Development.—The pathological changes occurring within the tissues of insect larvæ suffering from polyhedral disease have been frequently described. The most detailed descriptions, with the technical procedures involved, are those of Escherich and Miyajima (8) in their work on Wipfelkrankheit of nun moth caterpillars, of Glaser (3) in work with gipsy moth larvæ, of Prowazek (9) and Paillot (2) in studies on grasserie of silkworms. However, to understand the subject properly, it is necessary to again outline the principal features, incorporating those more recent detailed additions to our knowledge of the histopathology of the polyhedral affections. The histopathology of these diseases, as in so many diseases of humans and other animals, is intimately associated with diagnosis. For this reason alone, an accurate picture of the visible changes occurring within the tissues is necessary. The histopathology varies very little in different species, however, and we will confine ourselves to a discussion of the course of events in the two forms under consideration—the silkworm and the tent caterpillar. The histological technique employed was similar to that given in the work on gipsy moth wilt.

Diseased silkworms usually become jaundiced a few days before death and cease to eat. Tent larvæ show few recognizable external symptoms excepting loss of appetite. On death and shortly after both forms assume a shiny brownish color, are entirely flaccid and without any offensive odor. The cadavers are extremely difficult to remove without rupturing the skin and liberating the liquid contents. This is due to the fact that a few hours after death all of the tissues are in a state of disorganization. If a film from the milky fluid of a silkworm or the brown liquid of a tent larva is examined microscopically,

it will be found to contain the elements of disorganized tissues and myriads of polyhedral bodies of various sizes. In the silkworm the average polyhedra measure from 3–5μ in diameter, although individuals have been found as small as $\frac{1}{2}\mu$ and as large as 15μ. The tent wilt polyhedra are smaller, the average measuring about 2μ. They occur singly or in pairs, are very refractive and superficially resemble crystals. By manipulating the slide and cover slip, the bodies can be turned over, so that excellent views of all their faces can be obtained. Their shape varies as does their size and in silkworms 5, 6, 7, 8, and more faces are common. The corners are sharp and angular. In the smaller tent bodies the form is that of a polyhedron, with more or less rounded angles. They never assume the shape of a perfect sphere. As mentioned, the bodies are highly refractive, and on focusing seem to show a denser center differentiated from a somewhat lighter peripheral mass. Often, within the bodies, concentric layers like those of an onion are observable, demonstrating that development by accretion occurs. When pressure is applied, the polyhedra crack very readily into a number of pieces and often without the application of pressure the same fragmentation may be observed to occur somewhat more slowly. In the latter case a notch appears at one side of the polyhedron, which gradually lengthens into a line progressing slowly toward the other side, much like the cracking of ice. Usually, before the line has completely separated the two halves, other cracks appear, and soon the entire polyhedron is divided into a number of pieces, which may separate or stick together in a rosette-like fashion. The bodies have been carefully observed during the process of fragmentation, but nothing has ever been seen to escape from them. Fat globules and urate crystals have been confused with polyhedra by the inexperienced. However, the fat drops are perfectly spherical, stain with Sudan III and are soluble in ether. Polyhedra do not stain with Sudan III and are insoluble in ether. The urate and other crystals are of a totally different form, frequently show radiating lines, and react in their own characteristic manner.

A stained film of a worm, dead of grasserie or wilt, reveals polyhedral bodies, fat drops, urates and other crystals, cellular debris, hairs, setæ, food particles, and pigment granules. The pigment granules, so plentiful in a pigmented form like the tent caterpillar, must not be

confused with bacteria, to which they bear a superficial resemblance. As a matter of fact, a film from a larva recently dead is almost devoid of bacteria, and in many cases none can be found. If present, the larva has been dead for some time and bacteria have entered through various channels.

The polyhedra are very resistant towards stains in general, and usually color along the periphery only, unless the stain is applied for a long time following a mordant, or some rather drastic method is applied such as steaming with carbol fuchsin. When they react, they stain uniformly, or they may show structures that simulate small refractive granules and striations. The dark field reveals these granulations very well within the polyhedra. However, as previously mentioned, nothing escapes when the bodies are fragmented that could be correlated with the etiology of the disease.

The polyhedra are heavier than water, and consequently can be obtained in bulk by centrifuging aqueous emulsions of diseased material. By repeated washing and centrifuging, the fat and cellular debris, etc., can be eliminated and the bodies obtained in a fairly clean condition. They do not dissolve in hot or cold water, alcohol, ether, or chloroform, but are soluble in strong solutions of acids and weak solutions of alkalies when boiled in them. They do not blacken with osmic acid and do not color with Sudan III, and therefore contain no fat. Picric acid stains them yellow, showing that they contain protein material. In 1916, J. W. Chapman and the writer (1) found that the gipsy moth polyhedra meet all of the requirements of the nucleoproteins and regarded them as nucleoprotein crystal-like degeneration products of the disease. No further chemical work on these bodies was undertaken. Moreover, it does not seem likely that a more accurate chemical analysis of the bodies would throw any light on the problem.

Owing to the fact that dead larvæ disintegrate completely, living diseased material must be relied upon for sectioning. By infecting a large number of individuals and fixing one every few days, all stages of the disease can be obtained.

The most striking changes occur within the nuclei of the affected cells. If the untreated nuclei, of fat or blood cells, in early cases of the disease are carefully examined, many minute violently dancing

granules may be seen within. In 1915 (1), similar dancing granules were observed and described, and at that time regarded as either particles of degenerating chromatic and achromatic material or minute microorganisms. One finds such tiny granules in fresh films of all grasserie material whether dead or alive. They can readily be distinguished from pigment and other normal granules chiefly by their smaller size and greater vibration amplitude. Sections of diseased nuclei, prepared according to the method of Giemsa, demonstrate these little granules which stain red, are either single or double, and resemble tiny micrococci. Naturally, it is difficult to definitely decide the nature of these tiny particles, but the writer is now inclined to believe that they are degeneration products. The following experiment may be suggestive.

Tissue cultures of normal silkworm fat and blood cells were prepared on depression slides, under sterile conditions, using silkworm serum or Ringer's solution as a medium. These slides were held for two or three weeks, after which time various degrees of cellular degeneration of all the fat and some blood cells were obtained. Those blood cells placed near the periphery of the cover slip showed growth; they often multiplied and formed syncytia. This is a reaction produced under the influence of oxygen which filters through the thin layer of vaseline, as shown in 1918 (10). Within these normally multiplying blood cells, vibrating granules were not seen. However, all of the degenerating fat and blood cells showed minute, vibrating particles within the cytoplasm and nuclei indistinguishable from those seen in grasserie material. When fixed and stained, many of them seem morphologically identical to the granules found in disease. The writer does not offer this as absolute proof that the granules under the two different conditions are identical, but merely wishes to imply that when normal cells are permitted to degenerate they also demonstrate minute, violently vibrating granules.

In the normal silkworm and tent larval cell, the nucleus contains small round grains of chromatin distributed throughout the mass. The nucleoli are quite large and numerous; according to Paillot they always exceed ten in number within fat cells. Within the cytoplasm, according to this writer, the chondriome (mitochondria collectively) is represented by filaments distributed between the fat vacuoles.

Sections show that the polyhedra originate within the nuclei of the hypodermal, fat, tracheal matrix and certain blood cells. They are not found within the nuclei of the other tissue cells. The first indication of a diseased cell seems to be represented by a fusion of the chromatin grains and nucleoli. These form a large, dense, highly chromatophilic mass. This mass becomes speckled with minute, refractive bodies which Paillot believes originate from the chromatin grains. Within the clear area, left vacant by the condensation of the nuclear material, Giemsa's method demonstrates many little granules which bear a close resemblance to the dancing particles observed in the fresh preparations previously mentioned. Later, out of the clear nuclear zone, the polyhedra arise as very small individuals. At this stage the borders of the bodies are often faintly outlined by any stain used. As the inclusions increase in size, they become more and more refractive, do not stain at all finally, and the nucleus swells to an enormous size, sometimes measuring three times, or more, the diameter of the normal nucleus. This hypertrophy is due to the progressive increase in size of the inclusion bodies which simply fill the entire nucleus. All the polyhedra are in the same stage of development within an individual nucleus, but enormous differences in development occur between those of different nuclei. The small formative polyhedra are somewhat rounder than the larger individuals, which can partially be accounted for by the fact that, as the polyhedra grow, they become closely packed and press upon one another thus producing faces and angles. As the bodies grow and become more refractive, the little dispersed granules as well as the remains of the chromatin lump disappear and finally nothing remains but the nuclear membrane enclosing the polyhedra. Sometimes a portion of the chromatic mass persists till the nucleus disintegrates, but most frequently it vanishes before this event. Naturally such profound nuclear changes cause cytoplasmic disturbances. The earliest visible cytoplasmic changes, as pointed out by Paillot, occur within the mitochondria. The filaments disintegrate and are replaced by dispersed granules. Later, after the nucleus has become entirely incapacitated, the entire cell disintegrates and the inclusion bodies escape into the body cavity. It is remarkable how much injury the affected nuclei withstand before disintegration occurs. The disintegration of the cells beset with

polyhedra is probably independent of, although coexistent with, the lysis of all the tissues mentioned previously. Shortly after death all the tissues in the body disorganize, but it is difficult until the very last to detect any changes within the cells of the muscles, intestinal epithelium, Malpighian tubes, salivary and other glands, ganglia, nerves, gonads, etc.

The writer does not care to make any final pronouncement. However, with the evidence obtained from silkworms and tent caterpillars the safest view, in regard to the nature of the inclusion bodies, seems to be the one adopted previously (1913–16) in work on gipsy moth larvæ, namely, that the polyhedra are reaction bodies, arising in certain tissues as by-products of special nuclear changes produced by the virus.*

Normal and Pathological Blood Cells.—Since the blood has been used so much by various workers in determining whether an insect is suffering from polyhedral disease, and if so, how far the condition has progressed, a study of silkworm and tent caterpillar normal and pathological blood cell elements was undertaken. The cells were first studied in the fresh state, to observe whether pseudopodial formation with independent progression occurred, and to obtain a clear picture of the living cells. The same specimens were later fixed and stained by a variety of methods including those of Giemsa, Romanowsky, and Wright. The classification used by Paillot (11), and by Metalnikov and Gaschen (12) for insect blood cells was followed in so far as possible. Figs. 1 to 7, Plate I, represent sketches made from the dominant type of cell found (approximately 40–50 per 100 cells). Figs. 2 and 3 represent fixed and stained specimens, the others living cells. Fig. 7 shows one in the act of dividing. These cells are large elements each with a large nucleus and a considerable amount of clear cytoplasm. They show active ameboid movement and pseudopodial processes. These are the real leucocytes or phagocytes (macronucleocytes of Paillot) which engulf bacteria and foreign bodies.

The next most common type numerically (approximately 25–30 per 100) is represented by Figs. 8 to 11. These cells are generally

* Good microphotographs of nuclear lesions given in earlier work. See bibliography, especially under (3). Wilt of gipsy-moth caterpillars. J. Agric. Research, 1915, iv, 101.

somewhat smaller than the foregoing, are rounded or oblong, and never show any ameboid movement. The nucleus is quite small in comparison to that of the phagocytes and the cytoplasm has the appearance of being denser. As shown in Figs. 10 and 11 they often contain large granules resembling the eosinophilic granules in higher animals. These cells are the proleucocytes (micronucleocytes of Paillot).

In silkworms the lymphocytes rank next numerically (approximately 10–15 per 100). These are represented in Figs. 12 and 13, and are rounded elements each with a large nucleus, and a small amount of granular cytoplasm. They never show independent movement. Fig. 14 represents the spherule or mulberry corpuscle, so called on account of its superficial resemblance to a mulberry. This type of cell is not very common in silkworms, ranking fourth numerically (approximately 10–15 per 100). In tent larvæ they are exceedingly common, ranking second in abundance (approximately 25–30 per 100). They can be readily distinguished from the other blood cells by adding a little neutral red to the preparation. This will produce a picture showing curious, irregularly rounded cells with a peripheral zone of spaces filled with red staining spherules. The same cells are often encountered with the spaces empty, Fig. 15. The nucleus is large and centrally located. On crushing the spherule cells with their vacuoles filled, one obtains a picture, Fig. 18, which shows the scattered spheres, and the nucleus distorted and elongated by crushing. The spherule cells have no independent movement. They have often been mistaken for pathological cells and the cytoplasmic spherules for polyhedra.

Fig. 16 represents a type of cell not common, but which probably ranks fifth numerically in silkworms and tent caterpillars. This is the œnocyte-like or pseudo-œnocyte corpuscle, falsely called œnocyte. It is a non-motile, large, rounded or oblong cell with a dense, homogeneous cytoplasm and a small nucleus. It has a light yellow tint in the fresh state, and superficially resembles the stationary, large, glandular cell found around the spiracles and properly called œnocyte. Lastly, at times, giant cells are encountered. These are found in the blood of perfectly normal animals and are simply huge cells containing five, six, or more nuclei. Figs. 17 and 19 represent degenerating forms of unknown cells found in normal blood. Naturally, these various

types of cells vary somewhat in aspect; during metamorphosis when the phagocytes especially are loaded with metabolic products.

In diseased larvæ polyhedra develop within the nuclei of the phagocytes, proleucocytes, and lymphocytes; in those cells which are considered, by some workers, to represent stages or transitions of the same element. The inclusions are never found within the nuclei of mulberry corpuscles or œnocyte-like cells. We have never found them in giant cells. When a few small refractive bodies are found within the nucleus of a phagocyte, as shown in Fig. 20, it is rather difficult to decide whether infection has occurred or not. The writer has seen such pictures, but the larvæ subsequently failed to develop symptoms. When such nuclei are encountered as represented in Fig. 21 or 22, however, there can be no doubt that the insect will sooner or later succumb. As is the case within the other tissues, the polyhedra develop within the nuclei of the blood cells; when found in the cytoplasm, they have been phagocytized while free in the plasma. The origin of the inclusions in the blood cells is also preceded by a concentration of the nuclear substance and the formation of a central denser mass. Around this, one finds a zone in which refractive granules appear which gradually develop into polyhedra, and later completely fill the nucleus in the manner outlined for the other tissues.

During the last stages of the disease, when the tissues and blood cells begin to disorganize, myriads of polyhedra are found floating around free in the blood plasm. These free polyhedra, together with the liberated fat drops from the disintegrating fat body, give the blood of heavily diseased silkworms the appearance of milk. From the outline just given, it is evident that the blood is a fairly reliable index of the condition of a particular larva, and if two blood examinations are made on each animal, with an interval of a few days, one can be fairly certain whether polyhedral disease exists or not.

The Specificity of a Particular Virus for a Particular Host.

In 1915, the writer (3) pointed out that, although the gipsy moth reached this country in 1869 and became a serious pest in 1889, wilt among the larvæ was unknown prior to 1900. It can be safely stated that during a period of eleven years, before active suppression work was begun by the state of Massachusetts, not a single case of wilt was seen

or recorded, although entomologists and field men were continually on the lookout for parasites and diseases. At that time, it was known that wilt produced epidemics among gipsy moths in the Old World and Wipfelkrankheit, a similar disease among nun moth larvæ in Europe, had been recognized before 1892 when Tubenf (13), Tangl (14), Wachtl and Kornauth (15) made valuable contributions to the subject.

When wilt appeared in this country in epidemic form after 1900, etomologists and others began to wonder how the gipsy moth, heretofore such an extraordinarily healthy insect, acquired such a serious disease which proved of tremendous aid in the natural control of the pest. At that time it was recognized that a wilt occurred among some of our native American forms, especially among the apple tree tent caterpillars. The theory was advanced that tent wilt and gipsy wilt were identical, that the race of gipsy moths accidentally imported into America were immune, and that this immunity was gradually lost. Another theory assumed that the European Wipfelkrankheit and wilt were identical and that the former disease was imported into America after 1900 through the importation of trees and shrubs. A third theory assumed that all the polyhedral diseases were different, and that gipsy wilt was imported from European gipsy moths with plant shipments or with some of the insect parasites sent over here from Europe by the Federal Government after 1905.

No proof for any of these views has existed up to now, and to see whether some of these diseases, so similar superficially, were due to the same etiological agent or not, some experiments were undertaken. Three viruses were used, namely, the gipsy moth wilt virus, the tent wilt virus, and the silkworm grasserie virus.

Experiments 7 and 8, Table I, represent twenty tent larvæ fed with emulsified silkworm grasserie cadavers. In Experiments 9 and 10, the grasserie virus, which had been passed through a Berkefeld "N" candle, was inoculated into twenty tent larvæ. In Experiment 11, ten tent larvæ were fed with emulsified gipsy wilt cadavers, and in Experiment 12, ten tent larvæ were inoculated with a Berkefeld filtrate of the same virus. Although these viruses were known to be virulent for the hosts from which they were derived, not a case of wilt developed among the sixty tent larvæ.

Experiments 6 to 11, Table II, include infection experiments on silkworms (by feeding and inoculation) with the tent and gipsy moth

wilt viruses. No polyhedral disease developed among any of the sixty silkworms so treated.

These experiments show, therefore, that the grasserie, tent wilt, and gipsy wilt viruses are distinct and specifically limited to their hosts.

Immunity and Transmission of the Virus Through the Egg.

Experimenters have often noticed that a certain number of infected larvæ do not develop polyhedral infection and transform into moths. In 1915, the writer observed this immunity in his experiments, and also called attention to the same fact in his field studies on gipsy moth wilt. During a severe epidemic of wilt, in a heavily infested wood lot, thousands of gipsy larvæ die daily of the disease; and the body fluids everywhere soil the leaves which are eaten by others. Congregation in immense masses on the trees also occurs prior to pupation, and in such places the mortality is tremendous. An enormous reduction in the number of adult moths, and consequently in the number of egg clusters deposited will occur, but a complete extermination is never possible owing to the immunity of certain individuals in each generation.

Survival of a certain number of infected individuals again occurred during the progress of the present work. Ten tent larvæ and fifteen silkworms resisted disease after unquestionable infection. Rebouillon (16), in 1925, and Paillot (2), in 1926, reported the detection of grasserie in adult moths and Paillot can even detect infection within the eggs. If true this would constitute an extremely valuable discovery for the silk industry, because infected moths and eggs could be readily separated from the non-infected ones much after the very practical scheme devised by Pasteur for pebrine. However, we formerly repeatedly examined moths and eggs obtained from experimentally infected larvæ without finding any evidences of wilt. During field epidemics many female moths were dissected and examined for the presence of polyhedral bodies, but such examinations were invariably negative. Recently tent and silkworm moths and eggs were also subjected to examinations, but the writer could not obtain any positive evidence in this manner.

In 1925, eggs were obtained from some of the ten tent moths that survived the larval infection experiments previously outlined. Before death, or shortly after, all of the moths were microscopically examined

for polyhedra or other evidences of wilt. These examinations were negative. In the spring of 1926, the tent egg clusters were removed from ice, and "broken up" to separate the eggs which are held together by some mucilagenous substance. Twenty-five eggs were then submerged for ten minutes in a mixture of equal parts of 1–1000 corrosive sublimate and 95 per cent alcohol. This was done to kill any virus that may adhere to the outside of the chorion. Experience has shown that the wilt virus is rapidly destroyed by this treatment. The eggs were then washed in sterile water and placed on sterile filter paper in sterile glass phials, one egg to a phial. The phials were corked with cotton. On hatching each larva received cherry leaves selected from a region entirely free from tent larvæ. The leaves were further thoroughly washed before being tendered. After growing, each larva was transferred to a larger sterile bottle. Every possible precaution was taken to keep wilt infection from these larvæ, such as keeping them in a unit in which wilt had never occurred, and keeping away attendants who had been in contact with wilt. In spite of these precautions three of the twenty-five larvæ independently contracted wilt and died in the 4th and 5th stages. The others completed their development. Naturally, if these had been confined together the mortality would have been much higher. From this experiment it seems likely that the disease is transmitted from generation to generation through the egg in a small proportion of individuals. We are repeating and continuing this work and hope to extend it to silkworms, for which form we have as yet no concrete evidence.

The Resistance of the Polyhedral Viruses to Physical and Chemical Agents.

In 1918 (17), the effects of various physical and chemical agents on the gipsy moth wilt virus were reported. The effects of dry and moist heat, of low temperature, sunlight, desiccation, putrefaction, glycerin, alcohol, and carbolic acid, etc., were recorded. We have only had the opportunity to verify certain aspects of this work on the grasserie and tent wilt viruses.

SUMMARY.

1. This study concerns itself primarily with two polyhedral diseases, namely, grasserie of silkworms and wilt of tent caterpillars.

2. The viruses of tent caterpillar wilt and silkworm grasserie are

easily filterable through Berkefeld "V" and "N" candles. They can be passed through the "W" candles only with difficulty, and cannot be passed through the Pasteur-Chamberland "F" filters. These results may depend upon the size or formation of the filter pores; upon the flexibility or rigidity of the virus particles; upon the electrical charge, or upon some other factor.

3. Virulent filtrates are free from bacteria and polyhedral bodies and other visible agents that could be identified as etiologically important.

4. Under the dark field illumination with high magnification many minute, dancing granules can be observed in the filtrates. Similar granules are often seen free in the lymph of diseased larvæ, and within the cytoplasm and nucleus of pathological cells. These granules are interpreted as products of cellular disintegration.

5. Infection by the viruses occurs naturally through the mouth by feeding, but can also be experimentally reproduced by inoculation.

6. The period from infection to death varies from 4 to 24 days, with an average of 10 days. This variation probably depends upon the natural resistance of the host, upon the concentration of the virus, and upon temperature.

7. Detectable differences in size and shape exist between the polyhedral bodies of tent caterpillars and those of silkworms. Differences in size and shape exist within an individual host.

8. The polyhedral bodies can be freed from virus by repeated washing and centrifuging. The bodies so treated, entire or fragmented, are incapable of producing disease when injected into larvæ.

9. The polyhedra were subjected to certain microscopical, physical and chemical tests, but no evidence was obtained which could lead one to interpret these structures as living entities or as enveloping living material.

10. The polyhedra originate within the nuclei of the hypodermal, fat, tracheal matrix, and certain blood cells. The changes within the pathological cells are described and the intranuclear development of the inclusion bodies outlined.

11. The blood is a useful diagnostic index and can be used in following the progress of the disease. A detailed account of normal and pathological blood cells is given.

12. From all the evidence so far available it still seems necessary to regard the polyhedra as reaction bodies arising in certain tissues

as by-products of nuclear changes produced by the virus. They are of great diagnostic value.

13. Experiments are presented to show that silkworm grasserie, tent caterpillar wilt and gipsy moth wilt, so similar histopathologically are caused by distinct viruses. These viruses are absolutely specific.

14. It is shown that some larvæ are immune towards their specific virus.

15. It has so far been impossible to detect polyhedral disease within moths and eggs, but an experimental result is presented which seems to demonstrate that the tent virus is transmitted from generation to generation through the egg.

BIBLIOGRAPHY.

1. Chapman, J. W., and Glaser, R. W. A preliminary list of insects which have wilt, with a comparative study of their polyhedra. J. Econ. Ent., 1915, viii, 140.

Glaser, R. W., and Chapman, J. W. The nature of the polyhedral bodies found in insects. Biol. Bull., 1916, xxx, 367.

2. Paillot, A. Contribution a l'etude des maladies a virus filtrant chez les insectes. Ann. Inst. Pasteur, 1926, xl, 314.

3. Glaser, R. W., and Chapman, J. W. The wilt disease of gipsy moth caterpillars. J. Econ. Ent., 1913, v, 479.

Glaser, R. W. Wilt of gipsy-moth caterpillars. J. Agric. Research, 1915, iv, 101.

Chapman, J. W., and Glaser, R. W. Further studies on wilt of gipsy moth caterpillars. J. Econ. Ent., 1916, ix, 149.

4. Acqua, C. Ricerche sulla malattia del giallume nel baco da seta. Rendiconti dell Instituto bac. del R. Senola Super. di Agric. in Portici, 1918–19, ii, 243.

5. Aoki, K., and Chigasaki, Y. Immunisatorische Studien uber die Polyderkorperchen bei Gelbsucht von Seidenraupen (Zelleinschluss). Centr. Bakt. 1 Abt., Orig., 1921, lxxxvi, 481.

6. Komarek, J., and Breindl, V. Die Wipfelkrankheit der Nonne und der Erreger derselben. Z. Angenwandte Ent., 1924, Heft 10, 99.

7. Paillot, A. Sur l'etiologie et l'epidemiologie de la "grasserie" du ver a soie. Compt. rend. Acad. Sc., 1924, clxxix, 229.

8. Escherich, K., and Miyajima, M. Studien uber die Wipfelkrankheit der Nonne. Naturw. Z. Forst-u. Landw., 1911, ix, 381.

9. Prowazek, S. V. Clamydozoa. II. Gelbsucht der Seidenraupen. Arch. Protistenk., 1907, x, 359. Untersuchungen uber die Gelbsucht der Seidenraupen. Centr. Bakt., I. Abt., Orig., 1912, lxvii, 268.

10. Glaser, R. W. The aerobic nature of insect tissue. J. N. Y. Ent. Soc., 1918, xxvi, 1.

11. Paillot, A. Les maladies bacteriennes des insectes. Ann. des Epiphyties, 1922, viii, 190.

12. Metalnikov, S., and Gaschen, H. Immunite cellulaire et humorale chez la chenille. Ann. Inst. Pasteur, 1922, xxxvi, 233.

13. Tubenf, Carl V. Die Krankheiten der Nonne (Liparis monacha). Forstl. Naturw. Z., 1892, i, 62. Weitere Beobachtungen uber die Krankheiten der Nonne. Forstl. Naturw. Z., 1892, i, 277.

14. Tangl, Franz. Bakteriologischer Beitrag zur Nonnenraupenfrage. Forstw. Centr., 1893, xv, 209.

15. Wachtl, F. A., and Kornauth, Karl. Beitrage zur Kenntniss der Morphologie, Biologie und Pathologie der Nonne (Psilura monacha). Mitt. Forstl. Versuchsw. Osterr., 1893, Heft 16, 1.

16. Rebouillon, A. Sur la selection macroscopique et microscopique des papillons de vers a soie du murier atteints de la maladie de la grasserie. Compt. rend. Ac. Agric., 1925, xi, 744.

17. Glaser, R. W. The polyhedral virus of insects with a theoretical consideration of filterable viruses generally. Science, 1918, N. S., xlviii, 301

18. Glaser, R. W. A bacterial disease of silkworms. J. Bact., 1924, ix, 339. Specificity in bacterial disease with special reference to silkworms and tent caterpillars. J. Econ. Ent., 1925, xviii, 769.

EXPLANATION OF PLATE XVII.

SKETCHES OF NORMAL AND PATHOLOGICAL SILKWORM BLOOD CELLS.

Figs. 1–7. Normal leucocytes or phagocytes.

Figs. 8–11. Normal proleucocytes.

Figs. 12–13. Normal lymphocytes.

Fig. 14. Normal spherule or mulberry corpuscle treated with neutral red to show spherules in peripheral spaces.

Fig. 15. Normal spherule cell with peripheral spaces empty.

Fig. 18. Normal spherule cell crushed, showing discharged spherules, and elongated nucleus deformed by crushing.

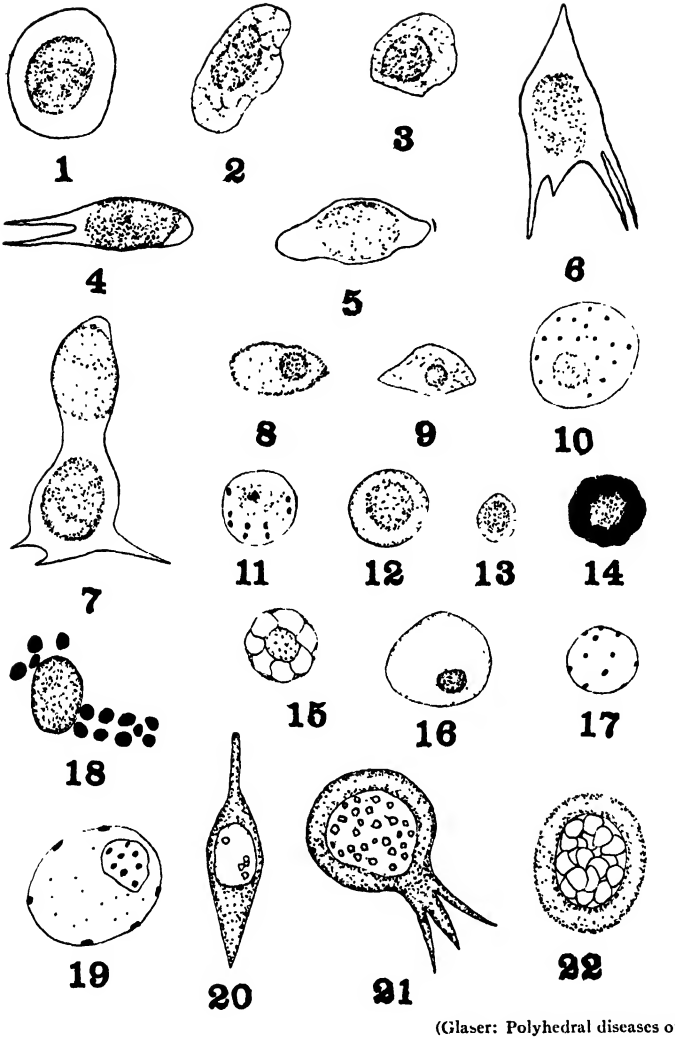
Fig. 16. Normal œnocyte-like cell.

Figs. 17 and 19. Degenerating forms found in normal blood.

Fig. 20. Leucocyte, at times found in grasserie infected larvæ with a few questionable inclusions within nucleus.

Fig. 21. Pathological leucocyte found in grasserie larvæ showing many small polyhedra within nucleus.

Fig. 22. Pathological leucocyte in grasserie larvæ showing entire nucleus filled with fully developed polyhedra.



(Glaser: Polyhedral diseases of insects.)

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EXPERIMENTS ON THE VISIBILITY OF THE POLYHEDRAL VIRUSES.

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In recent years the conception of the existence of filterable viruses as excitants of disease has been considerably extended.[1] It is now generally recognized that these agents, whatever they may be, operate in plants and insects as well as in the higher animals and man. But they are very elusive and it is questionable whether they may be seen microscopically; some of them most probably not. We have concentrated on this question of visibility with what we believe to be unusually favorable material, namely, the polyhedral viruses of insects, and in particular those which produce a "wilt disease" of gypsy larvæ and "grasserie" of silkworms. It has been claimed that these diseases are caused by a chlamydozoan[2,3] and by a *Borrellina*.[4] Previous work by one of us revealed no organisms of etiological significance in either.[5,6] We wish now to report briefly our comparison of diseased and normal blood made because the viruses are present in the former and absent in the latter.

Technique.

We employed many methods of fixation and staining and tried to devise new ones without finding a technique more satisfactory than

[1] Rivers, T. M., *J. Bact.*, 1927, xiv, 217.
[2] Wolff, M., *Mitt. Kaiser Wilhelm Inst. Landes. Bromberg*, 1910, iii, 69.
[3] von Prowazek, S., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxvii, 268.
[4] Paillot, A., *Ann. Inst. Pasteur*, 1926, xl, 314.
[5] Glaser, R. W., *J. Agric. Research*, 1915, iv, 101.
[6] Glaser, R. W., *Ann. Entomol. Soc. America*, 1927, xx, 319.

that of Giemsa. Various methods of vital staining were attempted but shed no light on the problem. Our optical equipment consisted of one of the latest Zeiss microscopes with lens combinations giving magnifications of from 900 to 2,400 diameters. In the examination of fresh preparations with different types of condensers a strong arc light was found indispensable. The condensers most frequently used were the paraboloid condenser which shows the reflections of colloidal particles down to 100 mμ in diameter, and the cardioid condenser (also called the cardioid ultramicroscope) which reveals the reflections of particles down to 50 mμ. The ultramicroscope of Siedentopf and Zsigmondy by which one may detect reflections of particles as small as 10–15 mμ was also employed.[7]

Qualitative Comparisons.

A representative protocol follows.

Blood from early cases of gipsy moth wilt and blood from early cases of silkworm grasserie were separately collected under sterile conditions. The blood from each insect was divided into two portions. With one of these from each diseased gipsy larva, five healthy gipsy larvæ were inoculated; and with one from each diseased silkworm, five healthy silkworms were likewise injected. The healthy gipsy larvæ were collected from a lightly infested tract of woods and examined for the presence or absence of polyhedral bodies according to the well known blood method. Only those larvæ entirely free from polyhedra were used. The silkworms which we employed had been reared in the laboratory, and since the entire stock was free from grasserie, no further precaution was necessary. All the inoculated insects subsequently died of either wilt or grasserie within the expected time, showing that the blood contained the infectious agent. Controls, inoculated with blood inactivated by heat, survived. The other portion of the infectious blood from each animal was studied immediately after being drawn in both the fresh and the fixed and stained condition.

The methods of Romanowsky, of Wright, and especially of Giemsa very often brought to light, in exceedingly thin films of the diseased blood, minute single or double coccoidal bodies colored pale pink or blue. These bodies could be best seen with a 1.5 mm. apochromatic

[7] International Critical Tables, New York, 1926, i, $\mu = \frac{1}{1,000}$ mm.; mμ = $\frac{1}{1,000,000}$ mm.

objective, and a 10 or 20 ocular. Beside these little stained particles nothing else was visible except blood cells, unstained polyhedra, and some debris. But when normal blood was prepared and studied in an identical manner the same type of stained particles was observed. Examination with the paraboloid or cardioid condensers of fixed and stained normal and diseased blood showed many minute particles of various sizes not revealed by ordinary illumination. These were not dye particles, because Giemsa's fluid is not colloidal and the formation of precipitates was avoided. With fresh material no evidence of independent motility of the particles was ever observed when care was taken to exclude air currents from the preparations, and when vibrations were reduced to a minimum. As the diseases progressed, and the tissues commenced to disintegrate, the particles in the blood increased numerically. Up to a certain point the condition then obtaining could be imitated in normal blood by permitting it to stand for a few days until the cells disintegrated and liberated much particulate matter. Heavily diseased blood, tissues, and dead larvæ were, however, useless for finer microscopic study, owing to the rapid final lysis of all the tissues with the discharge of protein particles, fat globules, pigment granules, urates, and various other substances.

From such comparisons made between normal fresh blood and infectious fresh blood, and between normal stained blood and infectious stained blood, we have come to the conclusion that no observable qualitative distinction exists between the various particles found in the normal and in the diseased condition in either wilt disease or grasserie, and further that none of these particles resemble minute cells or organisms.

Quantitative Comparisons.

If some of the particles within the diseased insects are etiologically important, they should numerically outrank similar particles found within the normal individuals, and for this reason we attempted to secure quantitative data. That such may be of value is indicated by the fact that, in most bacterial and other infections of insects, the living causative agents are usually very numerous. This is probably due to the open form of circulation. Paillot[8] points out that diseases

[8] Paillot, A., *Ann. Epiphyties*, 1922, viii, 265.

in insects caused by a limited number of microorganisms, which produce injury through soluble toxins, are as yet unknown. Many counts were accordingly made of small microscopic and ultramicroscopic particles in normal blood and in blood from early cases of grasserie. We present a protocol of one experiment out of twelve.

TABLE I.

Counts of Particles in Silkworm Blood Made with the Aid of a Cardioid Condenser.

The blood from three normal and three grasserie-infected silkworms was used. Two preparations were made from each worm and the particles in ten fields of every specimen were enumerated. Lens combination: 1.5 mm. objective, and 10 ocular.

Worm	Preparation	1	2	3	4	5	6	7	8	9	10
Grasserie blood.											
A	1	165	154	83	95	115	43	195	29	42	58
	2	10	22	35	75	28	45				
B	1	24	67	68	76	55	74	22	26	52	32
	2	8	6	9	11	8	2	9	11	4	7
C	1	33	40	22	13	21	36	45	20	75	21
	2	18	26	30	43	40	35	21	9	42	48
Normal blood.											
A′	1	160	45	39	55	53	22	89	41	27	46
	2	80	51	110	60	35	75	70	19	44	38
B′	1	8	30	50	20	25	30	15	53	88	24
	2	14	7	25	38	37	2	45	40	8	56
C′	1	26	14	52	19	30	63	21	47	17	35
	2	43	7	56	50	20	23	38	29	24	25

0.1 cc. of normal silkworm blood and 0.1 cc. of blood from a silkworm infected with grasserie by inoculation 4 days previously were each diluted 100 times with sterile, distilled water. The diluted blood was then centrifuged ½ hour at 1,000 R.P.M. to eliminate the blood cells, the larger particles, and the polyhedra. It was found in 1927,[6] that centrifuging the virus for 4 hours at 2,000 R.P.M. did not reduce its infectivity. 1 standard drop (0.1 cc.) of the clear upper fluid from each lot was then placed on the center of each of a series of scrupulously clean cover-slips.

These were floated over mercury and dried in air, to obtain a uniform distribution of the particles present. A diaphragm was inserted into the ocular to reduce to about one-half the field of vision and the particles were counted both microscopically, and ultramicroscopically (with the aid of the cardioid condenser). This was done for ten fields on each cover-slip. In each of the twelve experiments two cover-slips representing normal blood and two representing grasserie blood were used. The microscopic shapes of the particles and their ultramicroscopic reflections seemed identical as studied in normal and diseased blood, and the size varied from about 50–100 mμ to approximately 0.5μ depending upon the type of illumination employed.

The average microscopic particle counts of diseased and normal blood were naturally considerably lower, owing to lesser visibility, than the average ultramicroscopic particle counts in the same preparations. It is significant, however, that the same ratio was found between the number of particles in the diseased and normal blood when examined in these two ways.

A statistical analysis[9] of the determinations (Table I) shows that the average ultramicroscopic particle count for grasserie blood was 42.3 $\pm$ 3.3, and for normal blood, 40.2 $\pm$ 2.3. The standard deviation for grasserie blood was calculated to be 38.3 $\pm$ 2.4, and for normal blood, 26.8 $\pm$ 1.7. The difference in the variability was 11.4 $\pm$ 2.9, or 3.9 times the probable error. It seems, therefore, that there is no outspoken difference in the number of ultramicroscopically visible particles existing in normal and grasserie-infected blood, although the number in the latter is slightly the more variable as one would expect in the case of animals whose tissues are undergoing such rapid degeneration.

SUMMARY.

With the techniques employed we have not been able to detect any qualitative differences between the particles visible in normal blood and in blood from cases of wilt disease and grasserie. Of the two conditions we can speak more definitely in the case of grasserie, because this we have studied quantitatively as well, that is to say, the particles visible microscopically and ultramicroscopically have been counted without bringing to light any marked difference between

[9] Kindly made by Dr. John W. Gowen.

normal and diseased blood. This leads us to believe that the virus of wilt disease is probably invisible, and the virus of grasserie almost certainly so, when studied with the optical equipment that we have used, and that further evidence will be necessary before one can accept the chlamydozoa or the *Borrellina* as the active etiological agents. Although these two polyhedral viruses do not appear to be visibly particulate, it does not follow that other filterable viruses are not organized in this way. Each should be considered on its own merits.

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THE PROPERTIES OF THE BACTERICIDAL SUBSTANCE IN MILK.

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From the work of many it has been established that fresh raw milk will inhibit for a time the growth of a variety of organisms.

Thus Hesse (1) noted that raw milk suppressed the growth of *B. typhosus* and the cholera vibrio. Park (2) recorded a decrease in the number of organisms in milk stored at 42°F. for 24 hours, a moderate increase being found when the sample was kept at 50°F. The inhibitory property became less efficient when the milk was kept at room temperature. Rosenau and McCoy (3) likewise studied the phenomenon and concluded that there is diminution in the number of organisms during the first 8 or 10 hours of incubation with a rapid growth thereafter. The action was more prolonged but less intense at 15°C. Jones and Little (4) working with the mastitis streptococcus always recorded definite inhibition during the first 4 hours of incubation and frequently multiplication did not take place during 6 or 8 hours.

Many views have been advanced in explanation of the phenonenon. Most workers favor the opinion that the milk substance is identical with blood alexin and is directly derived from the blood. On the other hand Rosenau and McCoy suggest that the lower counts after incubation in raw milk are explicable on the ground of agglutination and he infers that phagocytosis by leucocytes contained in milk may be in part responsible for the decrease.

Stocking (5) suggests that the lack of adaptation for growth in milk of the organisms employed in the experiments may be responsible for the phenomenon. Others believe that milk contains a definite bactericidal substance. This view may be said to be supported by Heinemann (6), Chambers (7), Hanssen (8), and Jones and Little. Hanssen explains the bacterial growth-inhibitory principle on the basis of the presence of oxidizing enzymes which originate in the food and reach the udder from the circulation. Jones and Little regard the substance as one resembling alexin but originating in the udder and differing from blood alexin.

From the work of others and previous work done in this laboratory sufficient evidence exists that there is in cow's milk a substance which is capable of restraining the growth of certain bacteria for definite periods. Little is known concerning the properties of the growth-inhibitory principle. Heinemann showed that it was destroyed when boiled or heated to 60°C. for 30 minutes. Chambers noted destruction at 80° or 90°C. for 2 minutes. Hanssen records that milk heated at 63°C. for 20 minutes and 70°C. for 15 minutes still retained its inhibitory activity although 75°C. for 15 minutes inactivated the substance. Jones and Little found that 62°C. for 20 minutes failed to appreciably affect the substance although 65° or 70°C. for like periods slightly altered its effectiveness; 80°C. for the same period, or boiling for 5 minutes completely inactivated it. They also showed that whey from milk coagulated by rennet contained the inhibitory principle in practically the same concentration as the original milk.

With the aim of obtaining more information about the principle a number of observations were made.

Method.

Since the methods used in the previous work had proved satisfactory the same general procedure was adopted. The milk was drawn directly from the cleansed udder, chilled, centrifuged at high speed and thus largely freed of fat, and heated at 58°C. for 20 minutes. Heating is usually advisable to rid the milk of organisms originating in the udder. After chilling it was distributed in amounts of 1 cc. into sterile agglutination tubes containing a glass bead. The tubes were then inoculated with a standard loop of 16 hour broth culture, diluted 1:200, of the non-hemolytic mastitis streptococcus, and incubated. All tubes were shaken at half-hourly intervals during the observations. For control purposes a portion of the milk or whey was boiled for 5 minutes and distributed and inoculated in the same manner. The contents of each tube was plated after definite intervals with 10 cc. of 2 per cent agar prepared from veal infusion. The plate cultures were incubated for 24 hours at 38°C. and the colonies counted.

Time of Maximum Concentration.

It is known that the concentration of the substance in the milk of young cows may be as great as in older cows. However, it is not known how soon after parturition it reaches its maximum in the secretion. In answer to this question the following observation is cited.

Experiment 1.—The colostrum and milk from cows were tested. A sample was obtained daily and refrigerated at 2–3°C. until three were on hand. They were then tested. The milk and colostrum in this experiment were heated at 60°C. for 20 minutes before they were inoculated. The protocol in Table I represents the findings in one instance.

TABLE I.

The Effect of Colostrum and Milk during Early Lactation on the Mastitis Streptococcus.

	Streptococci present after incubation at 38°C.				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Day of parturition	1,152	1,404	11,456	Innumerable	Innumerable
" after "	960	768	1,536	15,296	92,160
2 days after parturition	960	896	7,168	69,220	Innumerable
Control, 3 samples combined and boiled	1,218	4,564	Innumerable	Innumerable	"
3 days after parturition	1,024	832	2,880	10,816	40,896
4 " " "	1,024	896	1,472	72,232	Innumerable
5 " " "	960	896	7,168	69,220	"
Control, 3 samples combined and boiled	1,218	6,592	Innumerable	Innumerable	"
6 days after parturition	1,088	1,024	2,368	9,408	34,560
7 " " "	1,216	894	1,152	3,392	25,792
8 " " "	1,152	1,216	960	2,048	28,800
Control, 3 samples combined and boiled	1,344	3,860	51,840	Innumerable	Innumerable

From the protocol given in Table I it is apparent that the inhibitory principle is present in the colostrum of the 1st day but is not quite as effective as after a few days. This is not surprising since colostrum is largely an accumulated product composed to a considerable extent of blood serum proteins. The inhibitory action of the blood derivative would be inactivated at the temperature (60°C.) to which the colostrum and milk were subjected before the tests. During the first 4 or 5 days the concentration in the secretion of the inhibitory principles

is more or less variable. After this time the results are more uniform. From the findings at later periods, not recorded in the protocol given, it is certain that the principle is present in about its maximum activity after the 6th or 7th day.

Distribution in the Quarters of the Udder.

It seems logical to assume that the amount of the inhibitory principle would be relatively uniform in the secretion from the various

TABLE II.

The Effect of Milk from Various Quarters on the Growth of the Mastitis Streptococcus.

		Streptococci present				
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Cow 82	Right fore quarter	576	704	704	1,152	37,440
	Left " "	704	768	640	1,792	57,600
	Right hind "	640	512	768	576	15,552
	Left " "	640	640	5,632	Innumerable	Innumerable
" 07	Right fore "	704	704	768	1,600	72,000
	Left " "	576	704	2,112	14,400	Innumerable
	Right hind "	704	576	1,088	3,136	86,400
	Left " "	576	576	2,048	57,600	Innumerable
All samples combined and boiled		640	5,312	115,200	Innumerable	"

quarters. That this is not altogether true is evident from the next experiment.

Experiment 2.—The milk from each quarter of two cows was drawn into separate bottles. It was chilled, centrifuged to free it from fat, and heated at 60°C. for 20 minutes; then distributed and inoculated with the usual amount of dilute broth culture. Plate cultures were prepared as usual and the plates counted after suitable incubation. The results are given in Table II.

From the evidence submitted in Table II it is clear that the concentration of the inhibitory substance in the secretion from various quarters varies considerably. In the case of Cow 82 milk from the right hind quarter completely inhibited growth during the first 6

hours and considerable inhibition was noted during 8 hours. The milk from the left hind quarter inhibited during the first 2 hours but not thereafter. That from the other quarters was more efficient in this regard but not equal to that obtained from the right hind quarter. The same is true of the milk of Cow 07, the milk from the right fore quarter being more inhibitory than that from the others. It would appear that the milk from the right half of the udder contained more of the principle than that from the left. The secretion from four other cows showed similar variations. One might suppose the difference to be due to such factors as a more liberal inflow of blood serum to certain quarters or to an expenditure of the principle upon bacteria within the udder. But when the serum content of the milk specimen is measured by serum precipitin marked differences are not apparent. Furthermore the milk from a quarter invaded with streptococci may be equally as efficient in inhibiting growth under experimental conditions as that from uninvaded quarters.

Reactivation of the Principle.

If the inhibitory principle in milk is of amboceptor-complement nature, then it should be possible to inactivate it by heat and restore the activity by the addition of a little fresh milk. In order to test this point the following experiment was devised.

Experiment 3.—Milk from a single cow was obtained as usual. After freeing of fat, it was distributed in sterile tubes in amounts of 9 cc., and all tubes heated at 58°C. for 20 minutes. After chilling, two tubes were heated at 60°C. for 2½ hours, two others at 80°C. for 20 minutes, and a fifth was boiled for 5 minutes. 1 cc. of the milk heated at 58°C. for 20 minutes was added to the contents of one tube of milk which had been heated at 60°C. for 2½ hours, and a similar amount added to one of the tubes heated for 20 minutes at 80°C. The various portions were distributed in the small tubes and inoculated and tested as usual. The complete series then comprised milk heated at 58°C. for 20 minutes; two lots of milk heated for 2½ hours at 60°C., to one of which active milk was added; two lots of milk heated at 80°C., one of which was activated; and the boiled milk. The results of the tests are given in Table III.

The experiment was repeated with similar results. It is evident that 60°C. for 2½ hours does not completely inactivate the milk. The effect of adding the active milk is readily apparent but cannot be

regarded as reactivation since the substance in the active milk, combined with that still left after heating at 60°C. for 2½ hours, would be sufficient to give considerable inhibition. The results with the series which were heated at 80°C. are in agreement with this view. In my hands this temperature has been the lowest at which the principle becomes completely inactive, yet the addition of active milk restores to only a slight degree the inhibitory effect. In other experiments it has been possible to show that as little as 10 or 20 per cent of fresh milk added to boiled milk will influence the multiplication

TABLE III.

Experiment on the Reactivation of Heated Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs	After 8 hrs.
Milk heated at 58°C. for 20 min.....	832	576	512	640	17,280
Milk heated at 60°C. for 2½ hrs.....	640	576	9,792	72,000	Innumerable
Milk heated at 60°C. for 2½ hrs. + 1/10 volume of 58°C. milk.....	704	576	4,032	21,880	"
Milk heated at 80°C. for 20 min.....	768	17,280	Innumerable	Innumerable	"
Milk heated at 80°C. for 20 min. + 1/10 volume 58°C. milk.....	704	14,400	"	"	"
Boiled milk.....	640	23,000	"	"	"

during the first 2 hours. It seems reasonable to assume then that the substance in milk is not inactivated by heat in the sense that complement is, but that heating sufficiently to impair the inhibitory action of the milk results in actual destruction.

Filtrability of the Principle.

Is it possible to pass the inhibitory principle through filters that hold back the formed elements of the milk? From previous work it is known that whey obtained by coagulation with rennet contains the

inhibitory substance. Since whey is readily filtered it was used in the following experiment.

Experiment 4.—Samples of milk from two cows, obtained as usual, were freed of fat, mixed, and heated at 58°C. for 20 minutes. To each 150 cc. of milk, 2.5 cc. of rennet solution (1 rennet tablet dissolved in 10 cc. of 0.85 per cent NaCl solution and passed through Berkefeld candle V) was added, and after suitable incubation the whey was collected and stored in the refrigerator at 3°C. overnight. Next morning 30 cc. portions were passed rapidly through Berkefeld candles V, N, and W under a pressure of 58 to 60 mm. The unfiltered and filtered portions and whey boiled for 5 minutes were then distributed and inoculated as usual. The results of the test are given in Table IV.

TABLE IV.

The Effect of Filtration on the Inhibitory Principle.

Whey	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Untreated	1,984	1,728	1,792	1,792	51,860
Filtered through:					
Berkefeld V	2,176	1,792	1,856	2,048	12,672
" N	2,176	2,688	11,520	Innumerable	Innumerable
" W	2,026	5,568	115,200	"	"
Boiled	2,026	5,204	Innumerable	"	"

The bactericidal principle contained in whey readily passes through the pores of a Berkefeld candle V, and its activity is not appreciably impaired. A filter of N fineness withholds most of it, inhibition being noted only during the first 2 hours. The W candle takes out almost all since whey passed through this filter behaves in general like boiled whey.

The retention of the principle by filters can conceivably be due to adsorption. It is known that the removal of casein from milk during rennet coagulation fails to remove the inhibitory substance from the whey. The agent then is not readily adsorbed by the casein. The same holds true for the fat. The results of some experiments with other adsorbents are worthy of record.

Experiment 5.—Finely ground kieselguhr, kaolin, bolus alba, and animal charcoal were washed repeatedly in distilled water and dried; then weighed in amounts

of 1 and 2.5 gm. and sterilized in 50 cc. centrifuge tubes. Milk from two cows was freed of fat and heated at 58°C. for 20 minutes. To specified amounts of the adsorbents, 10 cc. of milk was added and the tubes shaken vigorously. The tubes were refrigerated 1 hour, again shaken, and then centrifuged for 10 minutes. The supernatant fluids were distributed as usual, inoculated, and tested. Inasmuch as milk mixed with 2.5 gm. of adsorbent behaved in a manner identical with that containing only 1 gm., the results of adsorption with the larger amount only are given in Table V.

The data given in Table V are derived from two separate sets of observations. The tests in which kieselguhr and kaolin were used were done in duplicate with the milk from two cows, the results of

TABLE V.

The Effect of Adsorbents on the Bactericidal Property of Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk	704	512	576	576	576
" + kieselguhr	604	462	512	384	576
" + kaolin	704	576	512	512	512
Boiled milk	640	5,440	66,240	Innumerable	Innumerable
Milk	896	640	512	704	640
" + bolus alba	832	768	768	640	704
" + animal charcoal	832	1,024	37,440	Innumerable	Innumerable
Boiled milk	768	8,640	86,400	"	"

only one series being here given. The bolus alba and animal charcoal tests were made later and were likewise done in duplicate. For this reason control findings of two lots of untreated and two lots of boiled milk are given. All the adsorbents used are negatively charged. Those that failed to adsorb the substance are slightly acid (pH 6.2 to 6.5). The animal charcoal had a pH of 7.2.

It can be said that the inhibitory principle is not adsorbed by casein, fat, kieselguhr, kaolin, or bolus alba, although considerable is taken out by animal charcoal. In this connection it is of interest to point out that blood complement is removed by kieselguhr.

The Effect of Desiccation.

A further series of experiments was undertaken to determine whether the inhibitory principle would withstand desiccation and, for the purpose, in addition to the material prepared in the laboratory commercial dried milks were employed. Several methods were employed for desiccation. Whey or milk was placed in thin collodion membranes and hung in a current of warm air. Dr. Henry Simms of this Department dried certain material by means of low pressure distillation. But the most efficient method was *in vacuo* over sulfuric acid in the refrigerator. This last method usually took 6 or 7 days. The dried material went into solution readily.

TABLE VI.

The Presence of the Bactericidal Substance in Commercial Dried Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk powder A.....	768	8,640	72,000	Innumerable	Innumerable
" " " boiled 5 min.....	704	2,304	72,000	"	"
" " B.....	832	576	704	704	57,600
" " " boiled 5 min.....	640	2,432	46,080	Innumerable	Innumerable

Experiment 6.—13 gm. of two brands of commercial milk powder was dissolved in 87 cc. of sterile distilled water. The milk was then freed of fat by centrifugation and both products heated at 58°C. for 20 minutes, a necessary precaution since both contained organisms. They were then distributed, inoculated, and tested as usual. The results are given in Table VI.

The differences in the concentration of the principle in the two samples are sharp. Product A was as good a culture medium when heated at 58°C. as it was when boiled. It appears that the principle was completely destroyed during the drying process. Product B had a well defined bacterial inhibitory action which was destroyed by boiling.

With milk or whey dried in the laboratory the results showed that the inhibitory principle would withstand drying but they were not as

striking as with commercial product B. This is brought out in Experiment 7.

Experiment 7.—Fat-free milk heated at 58°C. for 20 minutes was distributed in thin layers in sterile wide mouth bottles and desiccated over H_2SO_4 *in vacuo*. Low pressure was maintained by the use of an oil pump twice daily. Between evacuations the jars were stored at a temperature of 3° or 4°C. Under these conditions 7 days was required for complete drying. The residue before use was dissolved in sterile distilled water and the product tested in the usual manner. Some of the material was tested as soon as possible and the remainder in the form of dry residue stored in the room and dissolved and tested 18 days later. The results are given in Table VII.

TABLE VII.

The Effect of Desiccation on the Bactericidal Principle.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Sample tested shortly after desiccation	640	896	576	4,316	72,000
The same boiled for 5 min	768	6,912	86,400	Innumerable	Innumerable
Sample after 18 days storage in the room	256	320	9,472	51,840	"
The same boiled for 5 min	320	9,216	72,000	Innumerable	"

The milk used in the experiment would completely inhibit growth for 6 hours and markedly influence it for 8 hours, but the dried preparation was not quite as efficient in this regard. Storage of the dried material at room temperature led to further inactivation. Desiccation by evaporation through collodion membranes over $CaCl_2$, or by low pressure distillation, led to no better results. Some of the substance always remained in the residue but never to the same concentration as in the original material and its activity deteriorated on standing.

If it were possible to obtain the substance in a concentrated form, considerably more could be learned of its properties. When one or two volumes of alcohol is added to whey and the supernatant and residue completely freed of alcohol by low pressure distillation, both

products, added to boiled milk, fail to inhibit growth. One may assume then that alcohol destroys the principle.

It was hoped that the agent adsorbed on particles of charcoal could be redissolved in more concentrated form. Several experiments were made in which milk was adsorbed with charcoal and the charcoal mixed with dilute acids and alkali, dilute phosphate solutions, physiological sodium chloride solution, and distilled water, and permitted to stand for several days in the refrigerator, the supernatant solutions finally being added to boiled milk and tested. It was possible to obtain a little inhibition with the extracts of weak alkali, phosphate, and sodium chloride, but the results obtained were not encouraging enough to warrant further work.

DISCUSSION.

Substances contained in a complex fluid like milk may be attached to or so closely associated with some of the other components that their true behavior may be masked. Granting this the inhibitory principle must yet be regarded as a definite constituent of cow's milk which is secreted with the colostrum and early milk. Other observations not reported in this paper indicate that the substance is present in themilk throughout the lactation period. It is a characteristic ingredient of the milk, being present, according to our experience, in the secretion of all cows though it is true that its concentration varies in different animals and may even vary in the milk from different quarters of the same udder.

It has already been stated that several views are prevalent regarding the nature of the principle. Stocking's contention that milk affords an improper medium for the test organisms is open to question in view of the fact that boiling milk for 5 minutes renders it an admirable medium. Further the organism employed throughout the test is an udder inhabitant and so far as can be learned exists only in milk. The action of phagocytes is ruled out in milk that is heated at 58° or 60°C. for 20 minutes and further by the results of the filtration experiments. Agglutination of the streptococcus was not found on microscopic examination. Some have supposed the principle to be blood alexin, others that it is a ferment derived from food, and yet others that it is an alexin-like substance originating in the udder.

The third view would seem to be the most reasonable. It is known that blood alexin loses its antibacterial action when diluted, and it must be greatly diluted in milk in the light of the fact that serum globulin is present in milk in small quantities only. The milk substance is much more resistant to heat than blood alexin. Unlike alexin once its activity is impaired by heat it cannot be reactivated by active milk. It is not adsorbed by the same class of adsorbents. Colostrum, which contains a large proportion of blood serum, inhibits bacterial growth even when heated to 60°C., a temperature sufficient to inactivate blood alexin.

The inhibitory substance in milk may serve to protect the udder from the growth of many types of organisms. The usual period of bacterial inhibition for which it is responsible corresponds roughly to the period between feedings by the calf which would empty the udder under natural conditions.

SUMMARY.

Certain of the properties of the bacterial agent in milk have been studied. The substance is present in the colostrum and milk of the first few days of lactation as well as later. Its concentration varies in the secretion from various quarters of the same cow. Its activity is diminished by heat and cannot be restored again by the addition of active milk. The principle is present in whey and readily passes through the coarsest Berkefeld filter although a considerable portion is retained by N candles. The finest filter (W) completely retains it. It is adsorbed by animal charcoal but not by kaolin, kieselguhr, or bolus alba. It can be desiccated and its presence has been demonstrated in one brand of dried milk.

BIBLIOGRAPHY.

1. Hesse, W., *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 238.
2. Park, W. H., *New York Univ. Bull. Med. School*, 1901, i, 71.
3. Rosenau, M. J., and McCoy, G. W., *Bull. Hyg. Lab., U. S. P. H., No. 41*, 1908, 449.
4. Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1927, xlv, 319.
5. Stocking, W. A., *Rep. Connecticut Agric. Exp. Station*, 1904, 89.
6. Heinemann, P. G., The kinds of bacteria concerned in the souring of milk, Chicago, 1903.
7. Chambers, W. H., *J. Bact.*, 1920, v, 527.
8. Hanssen, F. S., *Brit. J. Exp. Path.*, 1924, v, 271.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

I. SPONTANEOUS AND EXPERIMENTAL UDDER INFECTION.

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During the past four decades many outbreaks of scarlet fever attributed to contamination of milk supplies have been reported. This occurrence claimed considerable attention during the later '80's of the 19th century. Since then, although outbreaks attributed to milk have been recorded, the number has apparently declined. In certain particulars epidemics due to contaminated milk resemble each other. Their explosive nature—the bulk of the cases occur during a short period, usually within a week—is characteristic. The history that the sick have partaken of milk from a common source and the fact that the disease is not epidemic in other parts of the community having a different milk supply are equally significant.

The origin of milk-borne epidemics of scarlet fever has in the past led to much discussion.

Probably the most commonly held view is that of actual contamination of the milk by convalescents or persons actually sick with the disease. This view is supported by many recorded outbreaks in which persons known to have come in contact with scarlet fever or who were actually suffering from the disease milked the cows, mixed, handled, or bottled the milk. Among others, Hemenway (1) records a large epidemic attributed to milk thus contaminated. Other outbreaks regarded as milk-borne could be traced to actual cases who delivered the milk from door to door. Chalmers (2) reports such an outbreak. In addition the return of milk bottles from houses where scarlet fever was present was held responsible for the disease along particular milk routes.

The widespread and severe nature of certain outbreaks in England during the period from 1880 to 1900 led to investigations that directed attention to the cow as a source of infection. The difficulty of tracing human contamination strength-

ened the suspicion. Power (3) and Klein (4) as early as 1882 attributed one outbreak to the contamination of milk from a case of puerperal fever in a cow. Klein showed that cows inoculated subcutaneously with material obtained from the throats of human patients developed abscesses at the injection sites. Purulent material from such abscesses when injected into healthy cows also produced abscesses. Later Power (5), Cameron (6), and Klein (7) reported their findings in the Hendon outbreak in which they failed to find a human source of infection. Suspicion was directed to certain newly purchased cows suffering with a malady of the skin of the udder and teats. This they regarded as a specific infection transmitted by the hands of the milker. The condition was characterized by general constitutional disturbance, sore throat, discharge from the eyes and nose, vesicular eruptions of the skin of the buttocks and udder. They regarded the rupture of the teat vesicles as the source of the milk inoculation. Russell (8) encountered a similar outbreak in which 101 persons contracted scarlet fever; a malady similar to the Hendon disease affecting two cows was noted in the herd. A calf fed the milk of one of these developed a severe febrile reaction. Hill (9) likewise reported milk-borne scarlet fever; certain cows in the dairy from which the supply was drawn were found affected with Hendon disease. Hamar and Jones (10) also cited an outbreak in which a disease of the skin of cows similar to that noted by Power, Klein, and Cameron was present in the herd. They were inclined to attribute the human infection to the cows since the milk was known to be infective before human cases occurred on the farm. M'Fadyean (11) disagreed with them on the grounds that Hendon disease as a scarlatinal infection of cows is unproved and that the milker in whose family scarlet fever occurred may have been responsible for the epidemic through contamination. As a further criticism that Hendon disease is not a specific scarlatinal infection of cows he points out that although the disease was first recognized in 1882 and seemed to prevail for a year or two it had not attracted further attention until 1909 when a similar disease was reported.

It is apparent then that there are several views regarding the method by which milk may become infective: first, contamination of the milk during milking or handling; second, the return of contaminated bottles or utensils from the household in which the disease exists; and third, a scarlatinal disease of the skin and udder of cows from which discharges may enter the milk during milking.

With the change in the status of the streptococcus as the etiological agent of scarlet fever during the past few years, a fourth means of contamination becomes apparent. The findings of Savage (12), T. Smith and J. H. Brown (13), Davis and Capps (14), Brown and Orcutt (15), and Benson and Sears (16) in milk-borne epidemics of septic sore throat are of considerable interest in this connection. It

has been shown that *Streptococcus epidemicus* may be implanted in the udder and be shed in the milk in such numbers as to give rise to severe outbreaks of sore throat among those consuming the milk. That such is not unlikely in outbreaks of scarlet fever attributed to milk seems plausible. This is especially significant in many epidemics traced to dairies where no mention is made of clinical examination of the udder or bacteriological examination of the milk. The possibility of udder infection may have been overlooked.

Our problem concerns itself with udder infections with streptococci similar to those found in scarlet fever.

History of a Milk-Borne Epidemic of Scarlet Fever.

Through the courtesy of the New Jersey State Department of Health we learned of a sharp outbreak of scarlet fever in a small town. About 200 cases occurred. Of these 159 developed from May 20 to 25, 1927. The State authorities found that the bulk of the cases was confined to a certain milk route supplied by one distributor. Further information directed their attention to one of the farms supplying the dairy. On this farm a daughter had scarlet fever in March, 1927. In addition a young man employed as milker had visited his home shortly before the outbreak where there was a child sick of scarlet fever. Representatives of the State Department of Health made throat cultures from everyone suspected of contaminating the milk and obtained hemolytic streptococci in two instances from throats of men handling the milk at the distributor's. For these cultures we are indebted to Mr. J. V. Mulcahy, Chief of the Bureau of Bacteriology of the State Health Department. In addition he furnished us with a culture from the young man who milked the cows, but it was of the *viridans* type.

On May 25, Dr. I. H. Shaw, veterinarian for the State Department of Health, visited the farm and examined microscopically the milk sediment from each cow. He noted one chronic case of mastitis (Cow 11) and an acute injury of the teat of the left hind quarter (Cow 3). The milk sediment of Cow 3 contained leucocytes and cocci.

In the meantime the milk from this farm had been excluded from the supply and a pasteurizer installed in the distributor's with the

result that the epidemic rapidly subsided. At our suggestion, and through the courtesy of the State Department of Health, the farm was visited on June 4, 1927. Samples were drawn directly from the udder of each cow into separate sterile bottles.

Examination of Milk from Each Cow.

The samples were obtained on the evening of June 4, refrigerated at once, and plated late the same night. The normal appearing milk was plated in two dilutions, 1:20 and 1:100; that from abnormal quarters at 1:1,000 as well as the lower dilutions. All plates were prepared with 0.5 cc. of defibrinated horse blood and 10 or 12 cc. of melted agar. Thirteen cows comprised the herd. The udders of eleven were normal. The plate cultures revealed nothing of significance. Cow 11 had chronic mastitis of the left hind and right fore quarters. The plate cultures revealed non-hemolytic streptococci in enormous numbers both in the involved and apparently normal quarters. Cow 3 had a severe involvement of the left hind quarter. The teat had been injured, the quarter was swollen and could be milked with difficulty. The milk was yellow and of the consistency of heavy cream. All the blood in the plate containing as little as 1:1,000 cc. of milk was hemolyzed within 12 hours. The centrifuged sample revealed a large quantity of sediment consisting of packed masses of leucocytes and enormous numbers of short chained streptococci. After refrigeration for 12 hours the milk was further diluted and plated so that finally it was possible to estimate that it contained 345,000,000 streptococci per cc. Colonies sufficiently isolated were chosen for subculture. It may be said that the initial tests, such as those for the presence of capsules, the laking of blood in the test-tube, pathogenicity for rabbits, and a final pH of 5.0 in dextrose broth, indicated that this culture was of human origin. In addition to the human type a small proportion of the non-hemolytic bovine streptococci was found in the original sample.

The Spontaneous and Experimental Infection in the Cow.

Cow 3 was purchased by this Department and is hereafter referred to as No. 1452. 4 days after the first observation the quarter was swollen, firm, and the teat showed a healing scar. Yellow, purulent milk could be expressed only with difficulty. Bacteriological examination revealed relatively few hemolytic streptococci and enormous numbers of the non-hemolytic mastitis type. Evidently the bovine type noted June 4 had nearly replaced the hemolytic streptococcus. The inflammation continued in the quarter until the cow was slaughtered on June 23. During this time hemolytic streptococci were always present in small numbers and the mastitis type in enormous numbers.

On one occasion a single colony of the hemolytic type was obtained from the milk of the right hind quarter. This strain was identical in all respects with that obtained from the left hind quarter. Although the milk from the other quarters was plated frequently streptococci were not found.

On June 9, the right hind quarter was inoculated by means of a teat tube with 1/1,000,000 cc. of a 24 hour broth culture of the hemolytic streptococcus from the left hind quarter. No reaction occurred and examination of the milk during the next 3 days failed to show the organism.

On June 14, the right fore quarter was inoculated by means of a teat tube with 1/500,000 cc. of the hemolytic culture and the right hind quarter with 1/100,000 cc. of the same culture. 7 hours later the streptococcus could not be cultivated from the milk from either quarter. After 24 hours there was little to be detected clinically. The appearance of the milk was not greatly altered, and the quantity of sediment was not excessive but it contained leucocytes and a few diplococci. Plate cultures, however, revealed 25,600 hemolytic streptococci per cc. in the milk from the right hind quarter and over 1,000,000 per cc. in that from the right fore quarter. After 48 hours the right hind quarter was hot, the milk was yellow and contained large, irregular floccules. The right fore quarter revealed nothing abnormal except that the milk was yellow and flocculent. Samples from both quarters revealed an excessive amount of sediment composed of leucocytes and streptococci. The bacterial counts of the milk were: right fore quarter = 45,000,000 hemolytic streptococci per cc., and right hind quarter = 1,240,000 per cc. The next day 38,000,000 hemolytic streptococci per cc. were found in the milk from the right fore quarter and 1,240,000 per cc. in that from the right hind quarter. From this time onward the number began to decline until on the 14th day the milk from the right fore quarter failed to show streptococci while that from the right hind quarter revealed 26,000 per cc.

During the period of observation it can be said that the inoculation failed to produce well defined clinical disturbances in the inoculated quarters, although the milk was purulent.

The udder obtained from the abattoir at the time of slaughter revealed a pronounced atrophy of the left hind quarter characterized by severe degeneration of the secreting structures and hyperplasia of the interstitial tissue. The two inoculated quarters showed lesions of the mucosa of the lower portion of the udder involving the mucosa of the large milk ducts accompanied by a purulent exudate.

Since the spontaneous case of infection was complicated by injury and secondary infection with the usual type of mastitis streptococcus,

the question might be raised as to whether the factor of injury is a necessary precursor of infection. Further the experimental disease incited by inoculation failed to resemble the spontaneous disease. In order to throw more light on these questions a second series of inoculations was made.

Cow 1462.—A Holstein cow was injected by means of a teat tube inserted into the left hind quarter with 1/1,000,000 cc. of an 18 hour serum broth culture of the 6th transfer of the streptococcus obtained from the left hind quarter of Cow 1452. Plate cultures prepared from similar dilutions indicated that between 15 and 20 streptococci were injected. Plate cultures of the milk 7 hours after injection failed to show streptococci. 24 hours after injection the quarter still appeared normal. The milk contained only a little sediment composed of a few leucocytes and round cells and a few diplococci. The plate culture revealed 115,000 hemolytic streptococci per cc. After 31 hours the quarter was swollen, tense, hot, and

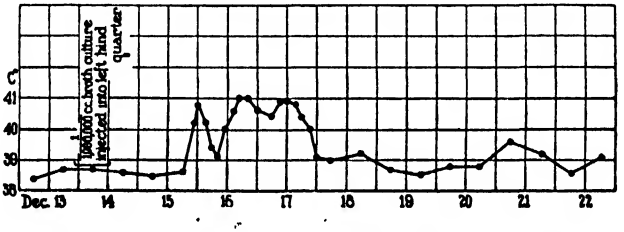

CHART 1. Temperature reaction, Cow 1462, following injection of left hind quarter.

painful. The milk contained an excess of fat but the amount of sediment was not excessive. There were 2,000,000 streptococci per cc. At 36 hours the quarter was greatly swollen. The milk was yellow and serous and contained large floccules. It coagulated on boiling. Plates revealed 8,320,000 streptococci per cc. 48 hours after injection there was a severe systemic reaction characterized by chills, fever (Chart 1), and depression. Swelling of the quarter was pronounced. The milk was seropurulent. It was estimated that 1,200,000,000 streptococci per cc. were being eliminated. On the 3rd day the quarter was distinctly reddened, the milk purulent, and 510,000 streptococci per cc. were recorded. A blood culture was negative. The cow had a fever. The reddening of the skin spread to the other quarters on the 4th day, persisted throughout the 5th day, and began to subside on the 6th day. It had disappeared by the 7th day. During this period the number of streptococci decreased until a minimum of 20,000 per cc. was reached. However they increased during the 7th, 8th, and 9th days, reaching the high point of 69,000,000 on the 8th day. There was a corresponding rise of temperature on the 7th and 8th days (Chart 1). By the 13th day their number

had declined to 2,000,000. From this time the acute inflammation gradually subsided with gradual atrophy and with a further decline in the secretion until on the 47th day only 25 or 30 cc. of purulent milk was obtained. Hemolytic streptococci were still present on this day. Chart 2 illustrates the number of streptococci per cc. of milk from the left hind quarter of Cow 1462 for 9 days following the inoculation.

It is to be noted that during the height of the attack the general reaction was severe, milk secretion was almost entirely suppressed, a fall from 8 pounds per milking to less than 1 pound being recorded.

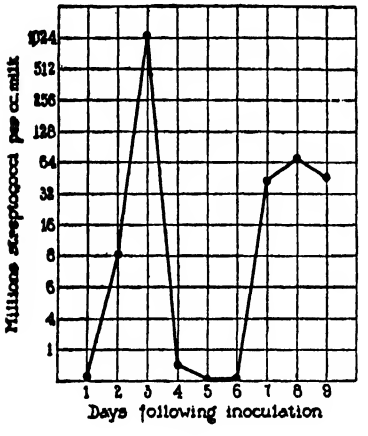

CHART 2.

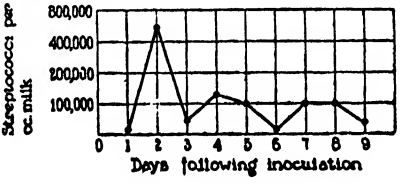

CHART 3.

CHART 2. Streptococci per cc. of milk during the first 9 days subsequent to injection of left hind quarter of Cow 1462.

CHART 3. Streptococci per cc. of milk during the first 9 days after injection of right hind quarter of Cow 1462.

.27 days after the left hind quarter had been artificially infected one-millionth cc. of broth culture was instilled into the lower cistern of the right hind quarter. It was estimated that approximately 70 streptococci were injected. 7 hours later there was nothing in the appearance of the milk to arouse suspicion and 1 cc. failed to reveal the streptococcus. After 24 hours the milk appeared normal. The sediment contained a few leucocytes and plate cultures revealed 1,800 streptococci per cc. 2 days following the inoculation the quarter failed to show abnormalities. The milk was yellow and thickened; when centrifuged the sediment comprised about 1/20 of the total volume. It was composed of masses of leucocytes, diplococci, and short chained streptococci. The maximum count of 556,000 streptococci per cc. was reached on this day. During the next 9 days the udder and milk were examined daily. Lesions of the udder were not detected

clinically. The milk was always purulent and the sediment at times made up 1/10 of the volume. The number of streptococci varied from day to day, the minimum count recorded during this period being 18,000 and the maximum 136,000 per cc. On the 21st day the milk was still purulent and flocculent and contained 20,000 streptococci per cc. Chart 3 is included for comparison with Chart 2. It indicates the number of hemolytic streptococci eliminated per cc. during the first 9 days following inoculation of the right hind quarter. The two charts afford a sharp numerical contrast. In the primary inoculation the maximum of over 1 billion was reached on the 3rd day following injection, with a rapid fall until the 6th day and a subsequent sharp rise on the 7th, 8th, and 9th days. Chart 3 illustrates the elimination of streptococci subsequent to injection of the right hind quarter. Here the peak of 550,000 was reached on the 2nd day with a sharp decline on the 3rd day and irregularity thereafter.

It is true then that the streptococcus isolated from the spontaneous infection was of sufficient virulence in spite of cultivation for 6 months on artificial media to incite a severe mastitis. During the acute inflammation there were marked systemic disturbances characterized by fever, increased pulse and respiratory rates, congestion of the conjunctiva, inappetence, suppression of milk, and stiffness of the joints. We regarded the animal as critically ill during this period.

In data from both the spontaneously infected and the experimental cow evidence exists that the primary attack so increased the general resistance that the secondary injection resulted in entirely different manifestations. Here only mild local disease resulted; although the streptococcus established itself, its multiplication never reached the maximum recorded in the primary attack.

It may be argued that the spontaneous case (No. 1452) referred to is an isolated example of such infection.* While this is true to a certain

* While these data were being assembled, Mr. Friend Lee Mickle, Director of the Bureau of Laboratories, Connecticut State Department of Health, sent three cultures of streptococci for examination. The interest of the Health Department had been aroused because of an outbreak of mild scarlet fever which appeared among the customers along a certain milk route. One of the cultures was isolated from the udder of a cow in the herd supplying the milk, another from the throat of the owner of the herd, and a third from a case of scarlet fever on the milk route. These proved similar in cultural characters, pathogenic properties, and antigenic affinities to those obtained by us from the udders of two cows. In all probability the particulars of this outbreak will be published by the Connecticut State Department of Health.

extent, nevertheless material obtained from another case of mastitis associated with a similar organism suggests that such infections may occur at any time. Although at the time of isolation the significance of the bacteriological findings in this spontaneous case was not realized, nevertheless the organism was regarded as a human type other than *S. epidemicus*. A brief statement concerning this case follows.

Cow 4262.—Milk drawn from all four quarters into a sterile bottle May 11, 1925. When plated it was found to contain 720 colonies per cc.; 25 per cent were large zoned, hemolytic colonies. May 19, 1925, mastitis of the right fore quarter was noted. The milk was purulent and flocculent. 77,000 hemolytic colonies per cc. were recorded. On May 22 and 23, the milk from all quarters was examined with negative results. The hemolytic streptococcus had been replaced by the bovine non-hemolytic type. The milk was again examined on May 27 and 28, and the later findings confirmed. Little of significance could be obtained from the history of this cow except that it had been in the herd for 3 years. During the preceding lactation periods many attacks of mastitis had been noted. During the 2 months prior to our examination four attacks of mastitis of the right fore quarter had been reported. The hemolytic streptococcus was definitely of the human type as proved by the usual tests and, as it will be shown later, closely resembled those isolated from Cows 1452 and 1462. No record is available which indicates that this animal was responsible for an outbreak of scarlet fever.

DISCUSSION.

Heretofore contamination has been regarded as the usual means of spread of scarlet fever by milk. The findings in regard to septic sore throat, however, indicate that udder infection with the human streptococcus is far more likely. The same may be said of milk-borne epidemics of scarlet fever.

Unfortunately our examination was conducted after the epidemic had subsided. It is true though that when the milk from this farm was withheld from the general supply and the general supply pasteurized the outbreak subsided. When we made our examination the owner maintained that the milk from the left hind quarter of the infected cow was "all right" although he had withheld it from the general supply. It can be argued, however, that such milk was fully capable of causing severe illness among the consumers provided it entered the general supply. If 1 quart of milk drawn at the time when the streptococci were most numerous was mixed with the herd supply, the actual

dilution in this instance would amount to 1:100 since there were 12 cows contributing about 100 quarts. Our maximum count indicated well over 300 million per cc. in the spontaneous case and over 1 billion in the experimental inoculation. Assuming that the infective product was again diluted at least a hundred times at the distributor's the number of streptococci in even a small quantity of milk would be relatively high.

Spontaneous Case 1452 and experimental Case 1462 are examples of extremely severe infections and they indicate that the organism was highly virulent for the cow. The infection obliterated for practical purposes the primarily involved quarter. However the resulting general resistance in both instances was insufficient to protect other portions of the udder from infection. This argues for a prompt exclusion from the milking shed of cows with involved quarters since the organism may gain entrance to normal quadrants without exciting severe reactions.

In sharp contrast to these cases is that of Cow 4262 in which the organism was known to be present in the milk on two occasions 8 days apart. It probably inhabited the udder during this period. However it disappeared and was replaced by a bovine type and was not found subsequently. That this cow failed to cause trouble to consumers can be explained on several grounds, (1) that the milk contained relatively few organisms, (2) that the milk was mixed with that of a large number of cows (50 or 100) and sold in a large city where a few cases of scarlet fever would attract little attention, and (3) that when the milk became abnormal it was eliminated from the supply. This case argues for a prompt investigation of the milk of all cows when milk-borne infection is suspected, since a few days delay in instances of this kind may be sufficient for the disappearance of the streptococcus from the udder.

The period of incubation is of considerable practical importance. In regard to this period little is known in spontaneous infection. In all the experimental inoculations there was little to arouse suspicion during the first 24 hours although streptococci could be readily detected in the milk. After this time abnormalities in the appearance of the milk were apparent. It must be borne in mind that in these experiments the organisms were introduced into the milk cistern, and

although the number of streptococci administered was small, in all probability in the spontaneous disease the infective dose would be even smaller; and unless contaminated material was introduced by teat tubes or other means the organisms would probably be deposited at the meatus or about the ends of the teats. This indicates a somewhat longer incubation period than that observed in the experiments.

SUMMARY.

The clinical and bacteriological findings in two cows the udders of which became infected under natural conditions with hemolytic streptococci of the scarlet fever type are discussed. One of the cows was found in a herd supplying raw milk to a small town where a milk-borne outbreak of scarlet fever had occurred a short time before. When small numbers of the streptococcus obtained from this case were injected into the udder of a normal cow severe mastitis accompanied by a well marked general reaction resulted. Evidence leads to the conclusion that a severe attack of mastitis due to this organism in one quarter does not sufficiently immunize the other quarters to protect them completely since the streptococcus can be readily implanted in them. The secondary infections were much milder than the original process.

We wish to acknolwedge our indebtedness to Mr. W. T. Eakins, Assistant Epidemiologist of the New Jersey State Department of Health, who furnished us with the history of the outbreak; also to Mr. J. V. Mulcahy who supplied us with cultures F. C. and M. B. obtained from the throats of milk handlers, and to Dr. I. H. Shaw for accompanying us to the farm and for other courtesies.

BIBLIOGRAPHY.

1. Hemenway, H. B., *J. Am. Med. Assn.*, 1908, ii, 1115.
2. Chalmers, A. K., *Brit. Med. J.*, 1894, i, 426.
3. Power, W. H., *Rep. Med. Off. Local Gov. Bd.*, London, 1882–83, **xii**, 63.
4. Klein, E. E., *Rep. Med. Off. Local Gov. Bd.*, London, 1882–83, **xii**, 63.
5. Power, W. H., *Rep. Med. Off. Local Gov. Bd.*, London, 1885–86, **xv**, 73.
6. Cameron, J., *Rep. Med. Off. Local. Gov. Bd.*, London, 1885–86, **xv**, 85.
7. Klein, E. E., *Rep. Med. Off. Local. Gov. Bd.*, London, 1885–86, **xv**, 90.
8. Russell, J. B., *San. J.*, Glasgow, 1888, **xii**, 70.

9. Hill, B., *Pub. Health*, 1890–91, iii, 487.
10. Hamar, W. H., and Jones, T. H., *J. Comp. Path. and Therap.*, 1909, xxii, 363.
11. M'Fadyean, J., *J. Comp. Path. and Therap.*, 1909, xxii, 340.
12. Savage, W. G., *Rep. Med. Off. Local Gov. Bd.*, London, 1906–07, xxxvi, 253; 1907–08, xxxvii, 359, 425; 1908–09, xxxviii, 33.
13. Smith, T., and Brown, J. H., *J. Med. Research*, 1915, xxxi, 455.
14. Davis, D. J., and Capps, J. A., *J. Infect. Dis.*, 1914, xv, 135.
15. Brown, J. H., and Orcutt, M. L., *J. Exp. Med.*, 1920, xxxi, 49.
16. Benson, R. L., and Sears, H. J., *J. Am. Med. Assn.*, 1923, lxxxiii, 1608.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

II. A STUDY OF THE SCARLET FEVER TYPE OF STREPTOCOCCI ISOLATED FROM THE UDDER OF THE COW.

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(Received for publication, March 9, 1928.)

Our first paper (1) dealt with the clinical manifestations resulting from invasion of the udder with a hemolytic streptococcus of probable human origin. It was shown that the culture isolated from the udder was capable on inoculation into the udder of a second cow of producing severe mastitis.

It was also stated that the ability of the streptococcus to lake blood in the test-tube, its pathogenicity for rabbits, and a limiting hydrogen ion concentration of pH 5.0 in dextrose broth differentiated it from the bovine streptococcus and indicated its human origin. The present paper deals with this phase of the question and a further correlation of these streptococci with those of the scarlet fever group.

Source of Cultures.

The cultures were drawn from several sources. Our own isolations from the udder of the spontaneous and experimental cases were regarded as the primary type. Those strains kindly furnished us by Mr. J. V. Mulcahy, Chief of the Bureau of Bacteriology of the New Jersey State Department of Health, who isolated them from the throats of milk handlers at the dairy, have been indicated as F.C. and M.B. Two cultures of scarlet fever streptococci of known origin were used, one (N.Y.V) furnished by Dr. A. R. Dochez, of the College of Physicians and Surgeons, Columbia University, and the other (Sc. 55) supplied by Dr. Anna F. Williams of the Department of Health of the City of New York. In addition three cultures of *Streptococcus epidemicus* are included in some of the comparisons. Of this type, C. 54 is the second bovine passage strain of the streptococcus isolated by Brown and Orcutt (2) during a milk-borne epidemic of septic sore throat. The others (744 and 4560) were obtained at various times by one of us (F.S.J.) from the udder of cows.

Morphological and Cultural Findings.

It can be said that little difference exists between the organisms isolated from Cows 1452, 1462, 4262, and the throats of F. C. and M. B. and the known scarlet fever strains. In all, chains of cocci up to 30 in number are not unusual. All are Gram-positive and all encapsulated. On the whole the capsules are considerably smaller than those of *S. epidemicus*. After 24 hours incubation in plate cultures containing 0.5 cc. of defibrinated horse blood and 10 cc. of agar prepared from veal infusion the surface colonies are round,

TABLE I.

The Fermentation Characters of the Streptococci Isolated from the Udder and from the Throats of the Milk Handlers.

Culture	Final hydrogen ion concentration in							Action on milk
	Dextrose	Lactose	Saccharose	Mannitol	Raffinose	Inulin	Salicin	
Cow 1452, L.H.Q.	5.2	5.2	5.3	7.2	7.6	7.6	5.3	Firm coagulation.
" 1452, R.H.Q. (before inoculation)	5.2	5.2	5.2	7.2	7.6	7.6	5.4	" "
Cow 4262, R.F.Q.	5.0	5.0	5.0	7.4	7.4	7.6	5.3	" "
" 1462, L.H.Q.	5.2	5.4	5.3	7.6	7.4	7.4	5.6	" "
Throat F.C.	5.2	5.2	5.0	7.2	7.6	7.6	5.3	" "
" M.B.	5.2	5.3	5.3	7.4	7.4	7.6	5.4	" "
Non-hemolytic mastitis streptococcus from Cow 1452, L.H.Q.	4.5	4.6	4.6	7.0	7.6	7.6	4.7	" "

flattened, translucent, and rarely exceed 1 or 1.5 mm. in diameter. They are surrounded by a sharp clear zone. The deep colonies vary in shape from round to biconvex and form the nucleus of beta hemolytic zones averaging 2.5 mm. in diameter. A further 24 hours incubation fails to increase the size of the colonies to any great extent although the hemolytic areas are increased to 3.0–3.5 mm. The colonies of *S. epidemicus* employed in the comparisons were larger, more sharply raised, and distinctly mucoid. The hemolytic areas were larger. The non-hemolytic streptococcus isolated from the udder of Cow 1452 is included in Table I for purposes of comparison.

To test the biochemic activities, all cultures were grown in fermented broth (pH 7.4), to which sufficient of the test substance had been added to make a 1 per cent solution, for 7 days. The results are recorded in Table I.

Table I indicates a close resemblance in the fermentation characters of the streptococci isolated from the spontaneous udder in-

TABLE II.

The Effect of the Cultures on Rabbits.

Rabbit No.	Weight	Intravenous injection—24 hr. broth culture	Result
	gm.		
1	1,010	1 cc. Cow 1452, L.H.Q., Culture A	Died. 28 hrs. Septicemia
2	2,050	1 " " 1452, " " B	" during night of 2nd day. Septicemia
3	1,105	1 " " 1452, R.H.Q., before inoculation	Died. 36 hrs. Septicemia
4	1,425	1 cc. Cow 1452, L.H.Q.	" on 4th day. "
5	1,575	1 " " 4262, R.F.Q.	Febrile reaction during first 7 days. Abscess right hock. Chloroformed on 10th day. Streptococci recovered from abscess
6	1,040	1 " culture from throat of F.C.	Febrile reaction during first 2 days. Bacteriemia during first 24 hrs. Chloroformed on 11th day. Abscess right hock. Vegetation on right auriculoventricular valve
7	1,130	1 " " " " " M.B.	Febrile reaction throughout period Bacteriemia during first 3 days. Chloroformed on 11th day. Abscesses both elbows. 4 vegetations on right auriculoventricular valve

fections and those obtained from the throats of the handlers at the distributor's. All attacked dextrose, lactose, saccharose, salicin, and coagulated milk whereas the other substances were not fermented. It is of interest to note that the passage of strain Cow 1452 through the udder of Cow 1462 failed to change its fermentation characters. The acid production of the non-hemolytic bovine streptococcus offers a sharp quantitative comparison.

Mention has been made of the pathogenicity of our strains for rabbits. As soon as feasible after the isolations rabbits were injected intravenously with 1 cc. of a 24 hour broth culture. The results are recorded in Table II.

The cultures from Cows 1452 and 1462 were more virulent for rabbits than the others. That from Cow 4262 behaved about the same as did those isolated from the milk handlers. These streptococci tended to localize in the joints and heart valves.

It will be noted that the preceding characters indicate human origin, since the bovine streptococcus produces more acid and is not pathogenic for rabbits in similar doses. They afford no specific differentiation between *S. scarlatinæ and S. epidemicus*, although by inference it might be said that, since Culture 1452 was identical with those obtained from the throats of the milk handlers during an epidemic of scarlet fever, all three were of the scarlet fever type.

Further comparisons were regarded as essential. Attempts were made to group the strains by their agglutination affinities. Even though the cultures were grown for long periods at room temperature, as suggested by Shibley (3), and suspended in 0.2 per cent NaCl solution made alkaline with NaOH, the results were inconclusive.

The recent contribution of Lancefield (4) for the differentiation of streptococci by means of precipitin offered a means of separation. Lancefield has shown that immunization for long periods with broth cultures of streptococci results in the production of precipitin. For antigenic purposes in conducting the tests the growth from a liter of broth culture is suspended in salt solution to which sufficient N HCl is added to make a concentration of N/20. The suspensions are then extracted in a water bath at 100°C. for ½ hour and neutralized with N NaOH. They are then freed of bacteria by centrifugation. When suitable quantities of such antigen are brought in contact with potent antiserum, the usual precipitin phenomena develop. When the fluids are mixed considerable precipitate is formed.

Rabbits were immunized by intraperitoneal injections of killed cultures followed by smaller doses of living organisms. For this purpose strains Cow 1452, F. C., Scarlet Fever N. Y. V, Scarlet Fever 55, and *S. epidemicus* Cow 4560 were used. Even after prolonged injection the sera of rabbits treated with strain Cow 1452 revealed only a weak precipitin. Scarlet Fever 55 failed to give rise to any

precipitin. A fair serum was obtained with N. Y. V and strong sera with F. C. and *S. epidemicus* 4560. In Table III we have recorded the reactions resulting from the addition of 0.15 cc. serum to 0.4 cc. antigen.

By means of Lancefield's procedure it was possible to divide the streptococci into two groups. Those associated with the milk-borne epidemic of scarlet fever—Cow 1452, Cow 1462, and F. C. and M. B.—

TABLE III.

Precipitation of Extracts of Streptococci with Various Antisera.

Streptococcic extract	Antisera			
	Rabbit immunized with			
	Culture Cow 1452	Throat culture F.C.	Scarlet fever streptococcus N.Y.V	*S. epidemicus* 4560
Cow 1452 L.H.Q.	+*	+++	+++	+−
" 1452 R.H.Q, before injection	+	+++	+++	+−
after injection	+	+++	++	+−
Cow 1462 L.H.Q.	+	+++	++	+−
Throat F.C.	+	++	++	+−
" M.B.	+	+++	++	+−
Cow 4262	+	++	++	+−
Scarlet fever 55	+	+++	++	+−
" " N.Y.V.	+	+++	++	+−
S. epidemicus Cow 54	−	−	+−	+++
" " " 744	−	−	+−	+++
" " " 4560	−	−	+−	+++

* + indicates moderate contact reaction with definite flocculation after mixing; ++, a well defined contact with considerable flocculent precipitation after mixing; +++, the maximum reaction.

behaved like the scarlet fever streptococci N. Y. V and 55. The culture isolated from Cow 4262 had a similar antigenic affinity. The three strains of *S. epidemicus* comprised a separate group. There was a slight tendency to precipitation, as indicated by the +− reactions, when the extracts from the scarlet fever group were treated with *S. epidemicus* serum, but for purposes of classification these can be ignored since the reactions indicated by ++ and +++ were so marked as to be unmistakable.

In the course of the further correlation it proved possible in two instances by means of intracutaneous tests on persons susceptible to scarlet fever toxin to show that culture Cow 1452 produced a toxin similar to that of scarlet fever streptococcus N. Y. V and that this toxin was neutralized by scarlet fever antitoxin.

The following protocols are submitted.

Cultures 1452 and N.Y.V were grown for 5 days in flasks containing a shallow layer of broth. The cultures were centrifuged and the supernatant fluid passed through Berkefeld candles of V porosity. Dr. Richard E. Shope of this Department, who had never had scarlet fever, volunteered as a subject. One of the writers (F.S.J.) also proved susceptible to the scarlet fever toxin. The filtrates were diluted in salt solution and the equivalent of 1/100 cc. of filtrate injected intradermally. The same amount of each filtrate was mixed with 0.05 cc. of scarlet fever antitoxin and injected. In all there were four injections, (1) scarlet fever filtrate, (2) scarlet fever filtrate + scarlet fever antitoxin, (3) culture Cow 1452 filtrate, and (4) culture Cow 1452 filtrate + scarlet fever antitoxin.

Subject R.E.S. responded severely at Sites 1 and 3. Site 2 was negative and Site 4 revealed only a slight reddening without swelling, the redness subsiding rapidly.

Subject F.S.J. responded most severely at Site 3, and had a definite reaction a Site 1. Sites 2 and 4 revealed some reddening which rapidly faded.

DISCUSSION.

The evidence seems clear that a streptococcus indistinguishable from the scarlet fever type may, under certain conditions, gain access to the cow's udder and there produce characteristic inflammatory changes.

That the streptococci isolated from the cows under study fulfill the criteria outlined for human streptococci is shown by their ability to lake blood in the test-tube, their final hydrogen ion concentration (5.0) in dextrose broth, and their pathogenicity for rabbits. Furthermore they may be differentiated from *S. epidemicus* by their antigenic complex when tested with specific precipitin. Extracts prepared in the manner described by Lancefield precipitated well with scarlet fever serum but failed to do so when mixed with *S. epidemicus* antiserum. The neutralization of the toxin of culture Cow 1452 by scarlet fever antitoxin is further evidence of similarity. As far as we could determine the passage through a second cow failed to markedly change the *S. scarlatinæ*-like character of the strains.

SUMMARY.

The streptococcus isolated from the udder of a cow on a farm where an outbreak of scarlet fever originated has been correlated with known scarlet fever strains. This streptococcus and another also isolated from the udder of a cow are indistinguishable in cultural characters and certain antigen affinities from *S. scarlatinæ*. Skin tests indicate that the strain isolated from the milk of the cow in a herd to which the scarlet fever epidemic was attributed produced a toxin which was neutralized with scarlet fever antitoxin.

We are indebted to Dr. Richard E. Shope of this Department for cooperation in making the skin tests.

BIBLIOGRAPHY.

1. Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1928, xlvii, 945.
2. Brown, J. H., and Orcutt, M. L., *J. Exp. Med.*, 1920, xxxi, 49.
3. Shibley, G. S., *J. Exp. Med.*, 1924, xxxix, 245.
4. Lancefield, R. C., *J. Exp. Med.*, 1928, xlvii, 91.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

III. THE INFLUENCE OF MILK ON THE GROWTH OF SCARLET FEVER STREPTOCOCCI.

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PLATE 38.

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The view usually held covering milk-borne epidemics of scarlet fever is that the streptococci originate in the throats of the milk handlers and through contamination gain access to the supply. It is difficult to explain on such grounds the heavy incidence of infection among the consumers of pooled supplies since relatively few organisms would gain access to limited amounts of milk, and when this was mixed with other milk the dilution would be so great that the probable incidence of human infection would be relatively small. It might be argued that the contaminating streptococcus would multiply rapidly in the milk provided the temperature was favorable and that the product reaching the consumer would thus contain large numbers of the streptococci. But to explain severe outbreaks of scarlet fever through milk contamination one would have to assume that the organism gained access in goodly numbers and multiplied rapidly. However certain experiments here to be reported indicate that streptococci of the scarlet fever type are acted upon adversely by milk.

During the observations in connection with the artificial inoculation of Cow 1462 with a streptococcus of the human scarlet fever type a peculiar phenomenon was observed. It was customary to plate the milk from the involved quarters after logarithmic dilutions in salt solution, the first plate of a series containing a 10^{-1} dilution, the second a 10^{-2}, until to the final plate milk diluted 10^{-6} was added.

Toward the end of the observation, when the numbers of streptococci per cc. were not excessive, it was noted that the first plate culture contained streptococci in smaller numbers than one would expect from the number found in the higher dilutions. Furthermore the colonies were very small with correspondingly small hemolytic zones; yet when such colonies were subcultured and replated colonies of the usual size with well defined hemolytic zones developed. Two explanations suggested themselves, first, that the colonies were too numerous in the plate, and second, that an inhibitory substance had developed in the quarter which was active in the 10^{-1} dilution but not in the 10^{-2} dilution. Further observations indicated that overcrowding was not responsible for the change in the character of the colonies. The inhibition appeared to be a property of the milk and as such worthy of more careful study.

EXPERIMENTAL.

The cultures employed have been described in the preceding papers. They were carried for stock purposes on agar slants to which a few drops of defibrinated horse blood had been added. For the experiments transfers were made into veal infusion broth and incubated 16 hours. Inasmuch as the rest of the procedure varied, some details of the individual experiments are given separately.

Experiment 1.—Milk from individual quarters of Cow 1462 was drawn directly into sterile bottles and after chilling freed of fat by centrifuging. That from the quarters artificially infected with the hemolytic streptococcus was combined and portions of this milk and of that from an uninfected quarter were heated at 60°C. for 20 minutes, while other portions were not heated. In addition the cow was bled on the day before the experiment and the serum collected.

The inhibitory activities of the milk and serum were then tested in the following manner. Serum or milk was added in amounts of 0.5, 0.25, and 0.1 cc. to Petri dishes containing 0.5 cc. of defibrinated horse blood. A loop of dilute broth culture of the streptococcus obtained from Cow 1462 was added to 10 or 12 cc. of melted agar cooled to 48°C. and the whole plated. The plates were incubated for 48 hours at 38°C.

Inasmuch as the unheated milk contained many native organisms giving rise to many colonies in the plates, the results were imperfect and for this reason they will not be considered in detail, although it was clear that even under such conditions the growth of the streptococcus was inhibited.

The results of the first experiment are given in Table I.

The fact seems established by the experiment that when milk from either the infected or normal quarters was added to plate cultures containing small numbers of hemolytic streptococci a distinct inhibition resulted. The colonies were smaller and their hemolytic activity greatly diminished or even entirely suppressed, as when 0.5 cc. of milk from the two involved quarters was incorporated in the culture. In the series in which blood serum was mixed and incubated

TABLE I.

The Influence of Blood Serum and Milk on the Size of Colonies and Hemolytic Activities of the Streptococcus from Cow 1462.

	Plate containing	
	cc.	
Blood serum	0.5	30 colonies varying from 1.5 to 1.8 mm. Hemolytic zones 4 mm.
	0.25	20 colonies average 1.8 mm. Hemolytic zones 4 mm.
Milk heated 60°C., from uninfected quarter (R. F.)	0.5	No growth
	0.25	6 colonies averaging 0.4 mm. Indistinct hemolytic zones 0.8 mm.
	0.1	5 colonies 0.4 to 0.6 mm. Hemolytic zones 1.25 to 1.75 mm.
Milk heated 60°C., from involved quarters, R. H. and L. H.	0.5	No growth in 24 hrs. 48 hrs., 5 tiny, non-hemolytic colonies
	0.25	30 colonies 0.25 mm. Hemolytic zones 0.6 mm.
	0.1	6 colonies 0.025 mm. Hemolytic zones 0.75 mm.

with the culture material there was no inhibition. One may infer that the inhibiting property was not derived from the blood.

It seemed possible that the organism employed might be unique in sensitiveness to the inhibitory action of milk. Furthermore the incorporation of as much as 0.5 cc. of milk to the blood agar plate might perhaps alter the nutritive character of the medium and thus prevent normal growth. The more elaborate procedure employed in Experiment 2 was devised to control these features.

TABLE II.

The Effect of Milk on the Character of the Growth of the Scarlet Fever Streptococcus.

Culture	Milk	Plate containing	
		cc.	
F. C.	R. F., filtered	0.5	No growth 24 hrs. 48 hrs., 10 colonies, tiny, surrounded by faint hemolysis
		0.25	384 colonies, average 0.4 mm. Hemolytic zones 0.75 mm., indistinct
		0.1	345 colonies, average 0.6 mm. Hemolytic zones 1 to 1.25 mm., clear
	R. F., heated 58°C. 20 min.	0.5	320 colonies less than 0.25 mm. Hemolytic zones barely perceptible
		0.25	384 colonies average 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	350 colonies, average 0.6 mm. Hemolytic zones 1 mm.
	R. H., filtered	0.5	325 colonies barely visible × 9. Non-hemolytic
		0.25	400 " " " × 9. "
		0.1	384 colonies, average 0.9 mm. Hemolytic zone 3 mm.
	R. H., boiled 5 min.	0.5	428 colonies, average 0.9 mm. Hemolytic zone 3 mm.
	None		384 colonies, average 0.8 mm. Hemolytic zone 3 mm.
Scarlet Fever 55	R. F., filtered	0.5	90 colonies visible × 9. Non-hemolytic
		0.25	576 colonies, less than 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	580 colonies, average 0.5 mm. Hemolytic zones 2 mm.
	R. F., heated 58°C. 20 min.	0.5	No growth
		0.25	640 colonies less than 0.25 mm. Hazy hemolytic zones 0.5 mm.
		0.1	576 colonies 0.25 mm. Clear hemolytic zones 0.75 mm.
	R. H., filtered	0.5	No growth
		0.25	30 colonies visible × 9. Non-hemolytic
		0.1	179 colonies 0.25 to 0.3 mm. Clear hemolytic zones 0.6 mm.
	Boiled 5 min.	0.5	428 colonies 0.8 to 1 mm. Clear hemolytic zones 3 mm.
	None		284 colonies 0.8 mm. Clear hemolytic zones 3 to 3.5 mm.

Experiment 2.—Culture F. C. from the throat of a milk handler and Scarlet Fever Streptococcus 55 were employed in this experiment. Milk in separate bottles was obtained directly from the right fore and right hind quarters of Cow 1462. It was chilled and freed of fat. One portion of each was filtered through Berkefeld candle V, another lot heated at 58°C. for 20 minutes, and the remainder boiled for 5 minutes. The various lots in amounts of 0.5, 0.25, and 0.1 cc. were incorporated in the plate cultures. The effect on the growth of both strains is recorded in Table II.

The results of Experiment 2 resemble those of Experiment 1. 0.5 cc. of milk, filtered or heated at 58°C. for 20 minutes to rid it of native bacteria, proved sufficient to inhibit or entirely suppress the growth of the scarlet fever streptococcus. In certain instances although colonies of the organism developed in the Petri dishes no hemolysis occurred; nevertheless when such colonies were subcultured and replated in the blood agar mixture characteristic hemolytic colonies developed. The control to which 0.5 cc. of boiled milk was added failed to show appreciable inhibition.

Two series of the plate cultures were photographed. In order to afford a proper comparison they were magnified about 5 times. Figs. 1 to 5 are the photographs of the plate cultures of Strain F. C. without milk (Fig. 1), with boiled milk (Fig. 2), and the series in which 0.5, 0.25, and 0.1 cc. of filtered milk was added (Figs. 3, 4, 5). Figs. 6 to 10 show the effect of mixing milk heated at 58°C. for 20 minutes with the cultures of Scarlet Fever 55. Photographs of the two controls, one without milk and the other with boiled milk, are included for comparison.

It might be argued that the milk from Cow 1462 contained some immune property acquired as the result of partial recovery from infection. To test this possibility Experiment 3 was devised.

Experiment 3.—Milk was obtained directly from the udder of five normal cows chosen at random from those of a large herd. It was mixed and when freed of fat a portion was filtered, another heated at 58°C. for 20 minutes, and a third lot boiled for 5 minutes. It was then added in the usual amounts to the Petri dishes and its effect on cultures of Strain F. C. and Scarlet Fever V noted.

It was found after 48 hours incubation that the growth of Culture F. C. in the plates containing 0.5 cc. of either filtered milk or milk heated at 58°C. was completely inhibited. In plates containing 0.25 cc. either nearly complete inhibition occurred, or the colonies were too small to be seen with the unaided eye. Even

as little as 0.1 cc. of milk greatly diminished the size of the colony and the zone of hemolysis. The same could be said of Scarlet Fever V. In both series the addition of 0.5 cc. of boiled milk failed to diminish the number or size of the colonies although the hemolytic zones were a little smaller than in the control cultures which were made without milk.

It appears certain then that there is a natural inhibitory substance in milk which passes through a Berkefeld filter V and is not greatly injured when milk is heated at 58°C. for 20 minutes but inhibits the growth of streptococci of the scarlet fever type in plate cultures. The inhibition depends on the concentration of the milk but even when this is diluted as much as 1:100, as in the series in which 0.1 cc. of milk was mixed with 10 or 12 cc. of agar and 0.5 cc. of blood, its effect is readily visible.

Since cow's milk contains a principle which is inhibitory even when mixed with the culture medium, it seemed probable that exposure of the streptococci to the direct action of undiluted milk might result in definite destruction. To test this point milk was inoculated with the streptococcus and plated, in the manner outlined by Jones and Little (1), in the hope that the rate of growth or destruction could be measured. As might be expected from the preceding experiments, milk even when inoculated with 3,000 or 4,000 streptococci per cc., failed to show growth when plated in amounts of 1 cc. It was necessary to change the methods considerably before decisive experiments were obtained.

Experiment 4.—Milk from five cows chosen at random was mixed and freed of fat by centrifugation. A portion was heated at 58°C. for 20 minutes and the remainder boiled for 5 minutes. Both lots were then distributed into sterile agglutination tubes in amounts of 1 cc. The tubes were then separated into two groups each containing an equal number of tubes of the pasteurized and the boiled milk. Each tube of one group was inoculated with 1 loop of Strain F. C. diluted 500 times in broth. Those of the other group received a similar inoculation with Scarlet Fever V. Plate cultures were made by adding 0.25 cc. of milk to uniform amounts of blood and agar. Initial plates were poured and others after various intervals of incubation. The results of this experiment are recorded in Table III.

While there is some irregularity in the results recorded in Table III, the influence of the milk is evident. It is clear that fresh milk heated to 58°C. for 20 minutes actually prevents multiplication of

scarlet fever streptococci during incubation periods ranging from 2 to 48 hours. From the protocol it appears that the milk probably destroyed both strains of streptococci after an interval of 4 or 6 hours. To determine whether this was actually the case required further experimentation. It seemed possible that the organism subjected to the unfavorable influence of the milk heated at 58°C. was incapable of growth when plated because of the fact that the culture medium

TABLE III.

The Effect of Undiluted Milk on the Scarlet Fever Streptococcus.

Culture	Milk	Colonies developing in plate cultures containing 0.25 cc. milk						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F C.	Heated 58°C.	No growth	243 Visible × 12	256 Visible × 12	No growth	No growth	No growth	No growth. Reaction pH 6.6
F. C.	Boiled	409	4,708 Whole plate hemolyzed	72,000	Innumerable	Innumerable	Innumerable	Coagulates on boiling. pH 5.2
Scarlet Fever 55	Heated 58°C.	384	154 Visible × 12, non-hemolytic	No growth	No growth	No growth	No growth	No growth. pH 6.6
Scarlet Fever 55	Boiled	512	7,488	86,400	Innumerable	Innumerable	Innumerable	Coagulates on boiling. pH 5.4

contained 0.25 cc. of the inhibiting milk. This factor, added to the effect of previous exposure, might still be insufficient to kill the organism although suppressing its multiplication. That the same milk when boiled for 5 minutes was well adapted as a culture medium is obvious in the protocol, since multiplication was noted throughout the series.

In the next experiment only a trace of milk was added to the plate cultures.

Experiment 5.—Milk was obtained from the same cows as in Experiment 4. It was handled in a similar manner. After distribution in agglutination tubes in amounts of 1 cc., each tube was inoculated with a loop of broth culture diluted 500 times. Each tube before plating was centrifuged at high speed for 15 min-

TABLE IV.

Streptococci Surviving after Incubation in Milk.

Culture	Milk	Colonies developing in plate cultures						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F. C.	Heated 58°C. 20 min.	205	5	11	11	Sterile	Sterile pH 6.6	Sterile pH 6.6
F. C.	Boiled 5 min.	218	8,960	86,400	Innumerable	Innumerable	Innumerable pH 6.0 Thickens on boiling	pH 5.4 Coagulates on boiling
Scarlet Fever V	Heated 58°C. 20 min.	217	205	192	205	77	11 pH 6.6	Sterile pH 6.6
Scarlet Fever V	Boiled 5 min.	218	4,992	46,080	Innumerable	Innumerable	Innumerable pH 6.0	pH 5.6 Coagulates on boiling

TABLE V.

The Effect of Milk on Scarlet Fever Streptococci at 3° and 4°C.

	Before refrigeration	After refrigeration	
		24 hrs.	48 hrs.
Culture F. C. in milk heated 58°C. 20 min	205	24	12
" " " " boiled milk	218	300	412
" Scarlet Fever V in milk heated 58°C. 20 min	217	156	102
" " " " " " boiled milk	218	130	166

utes. The bulk of the milk was drawn off and 1 cc. sterile salt solution added and thoroughly mixed. The mixture was then withdrawn and added to the Petri dishes. In this way it was hoped that so little milk would be added to the medium that its effect would be negligible. The majority of the tubes were incubated

and plates prepared at indicated intervals. Some of the tubes were refrigerated at 3–4°C. and their contents plated by the same method after 24 and 48 hour intervals. The colonies were counted after an incubation of 48 hours in the blood agar medium. The results are given in Tables IV and V.

It is evident that the scarlet fever streptococcus fails to multiply in mixed milk provided the milk has not been heated sufficiently to destroy the inhibitory substance. It is also true that the principle in milk actually destroys the organism. The lethal effect is most marked at 38°C. At this temperature Culture F. C. was killed after 8 hours incubation. Scarlet Fever Streptococcus V was more resistant to the action of milk, since there was a more gradual diminution in the number of organisms, but after 48 hours none survived.

When the same cultures were exposed to the action of milk in the refrigerator Strain F. C. again proved more susceptible since only about 10 per cent of the streptococci survived for 24 hours, whereas culture Scarlet Fever V was not appreciably affected by the milk during a refrigeration of 24 hours or even 48 hours.

When the experiment was repeated at the temperature of the room, the number of streptococci was definitely diminished although not so greatly as at incubator temperature. The surviving streptococci failed to approach in number those implanted in milk and refrigerated.

DISCUSSION.

It can be said that milk heated at 58°C. for 20 minutes or filtered through the coarsest Berkefeld filter possesses the property of inhibiting the growth of the scarlet fever streptococcus. It also is true that the principle is sufficiently active to destroy certain of the organisms. That this phenomenon cannot be attributed to the lack of adaptation of the organism for growth in the food mixture represented by milk is amply shown by the behavior when boiled milk is used. It must be recognized that this activity is not specific for the streptococcus since it has been shown by others that other types of organisms are inhibited. However, it appears to be particularly potent for streptococci especially those of the scarlet fever type. The inhibitory and lethal effect of milk on the streptococcus is most marked at temperatures approaching that of the body. When artificially infected milk is stored at 3° or 4°C. more of the streptococci survive.

Since the evidence points to the udder as the source of origin of the inhibitory principle it is not surprising that the action of the latter should be most effective at a temperature about that of the organ in which it originates.

Milk added to plate cultures entirely prevents the development of colonies or so changes the appearance of the colonies as to make them unrecognizable. In many instances signs of hemolysis are not to be seen. Even when as little as 0.1 cc. of milk is added to the medium the surrounding hemolytic zone is so small that the colonies can easily be mistaken for the bovine type. These facts must be borne in mind when mixed milk is examined for human hemolytic streptococci.

Since the experimental evidence indicates strongly that milk, provided it is not heated at too high a temperature, will inhibit the growth or kill the scarlet fever streptococcus, the opinion that severe outbreaks of scarlet fever result from human contamination of milk must be viewed with considerable doubt. It might be objected that the experiments were artificial. But they were optimum conditions for the growth of the streptococcus and other organisms which would rapidly sour the milk under natural conditions were excluded. The change from a broth medium to one containing milk would appear to be no greater than that from a human throat to raw milk. That a few individuals may contract the disease through direct human contamination of milk is possible, but the occurrence of epidemics would imply a heavy inoculation of the milk. Infection of the udder of a single cow with the scarlet fever streptococcus and the resultant shedding of large numbers of the organisms into the milk,—phenomena recorded in our foregoing papers,—afford a more reasonable explanation of milk-borne epidemics.

SUMMARY.

The experiments indicate that milk filtered through a Berkefeld candle V or heated at 58°C. for 20 minutes when added to blood agar plate cultures interferes with the development of colonies of the scarlet fever streptococcus. The observed inhibition is proportional to the amount of milk. When the approximate milk dilution in the Petri dish is 1:20 or 1:25 growth of the organisms is completely suppressed or only a small proportion of non-hemolytic colonies develop.

As the amount of milk is decreased the colonies become larger and their hemolytic zones more pronounced, although even when the final dilution of milk reaches 1:100 or 1:125 only colonies easily mistaken for the narrow zoned bovine streptococci appear. The effects upon the surviving organisms would appear to be transient since both the non-hemolytic colonies and those with small zones manifest the original hemolytic properties when transferred to other media. When scarlet fever streptococci are added in small quantities to milk heated at 58°C. for 20 minutes and incubated growth is inhibited. If the period is prolonged the streptococci are killed. On refrigeration of such mixtures some of the streptoccci are killed but others survive the test period.

BIBLIOGRAPHY.

1. Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1927, xlv, 319.

EXPLANATION OF PLATE 38.

Magnification × about 5.

FIG. 1. Culture F. C. after 48 hours incubation in blood agar plate culture.

FIG. 2. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 3. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. filtered milk. There are no colonies of sufficient size to be detected at the magnification given.

FIG. 4. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.25 cc. filtered milk. Note the difference in size of colonies in this plate when compared with those in Figs. 1 and 2.

FIG. 5. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.1 cc. filtered milk. The colonies and hemolytic zones are larger than those in Fig. 4 but not as large as those in Figs. 1 and 2.

FIG. 6. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture.

FIG. 7. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 8. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. milk heated at 58°C. for 20 minutes. No growth visible at this magnification.

FIG. 9. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.25 cc. milk heated at 58°C. for 20 minutes. Note the small size of colonies and character of hemolytic zone as compared with Figs. 6 and 7.

FIG. 10. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.1 cc. milk heated at 58°C. for 20 minutes. The colonies and hemolytic zones are larger than those in Fig. 9 but not as large as those in Figs. 6 and 7.

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STUDIES IN BLOOD COMPOSITION OF ANIMALS UNDER PATHOLOGICAL CONDITIONS.

I. BRONCHO-PNEUMONIA IN COWS.

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INTRODUCTION

According to Myers (4) and others the development of severe pneumococcus pneumonia in human subjects often entails a more or less pronounced impairment of renal function, apparently secondary to the pneumonia. At the time of the crisis some increase in the non-protein nitrogen of the blood was found, due chiefly to a rise in the undetermined fraction. When the urea nitrogen exceeds 20 mgm. there is generally some creatinine retention. Recently McIntosh and Reiman (5) found that serious impairment of kidney function during lobar pneumonia was not encountered. Berger and Petschacher (1) and others showed also that in man there is in pneumonia a marked rise in the globulin fraction of the blood serum. In broncho-pneumonia most patients show a decrease in the blood chlorides before the crisis.

It seemed of value to find out what changes take place in the chemical composition of the blood in bovine pneumonia due to bipolar organisms (*B. bovisepticus*).

MATERIAL AND PROCEDURE

Eight cows, isolated from a herd on account of more or less high temperature and clinical symptoms of pneumonia of various degrees of severity, were bled from the jugular vein (40 cc.) and the blood plasma (oxalated) analyzed for glucose (Folin and Wu's method), chlorine (Whitehorn's method), non-protein nitrogen (Folin and Wu's method), uric acid (Benedict's method), creatinine (Folin and

TABLE 1.

Number	Date of introduction into herd	Date first symptoms were noted	Temperature	Symptoms	Termination
	1927	*1927*	°C.		
5	October 5	October 10	Normal	Atypical case, emaciation, inappetence, diminution in milk, slight lung involvement	Quarantine, 31 days
6	September 9	October 28	40 for 3 days, then relapse, after 7 days, 40–40.1 for 2 days	Typical case, dyspnea, bronchial breathing, inappetence, diminution in milk	Recovered, sick 29 days
7	October 7	October 25	41	Typical case, dyspnea, bronchial breathing, inappetence, diarrhea, diminution in milk	Recovered, sick 36 days
8	Native	November 8	40.3	Atypical, continual coughing, diminution in milk, emaciation, crepitant râles, constipation	November 25, slaughtered
9	October 5	November 3	39.3	Typical case, dyspnea, emaciation, constipation, lessened milk secretions, bronchial breathing	Recovered, sick 21 days
10	July 8	October 20	40.4	Typical case, dyspnea, crepitant râles, mucous membranes pale, rapid pulse, diminution in milk	October 27, sold to butcher. Carcass condemned. Autopsy: lobular pneumonia, fatty degeneration of liver
11	October 5	November 15	40.1–40.5 for 3 days	Atypical case, mild form of pneumonia	Recovered, sick 9 days
12	October 15	November 15	39	Atypical case, mild form of pneumonia	Recovered, sick 9 days

Wu's method), cholesterol (Bloor's method), and albumin, globulin and fibrin (Wu and Ling's method (8)). Control analyses were carried out on the blood plasma of four normal cows.

TABLE 2.

Blood Constituents in Normal and Pneumonia Cows.

(Figures per 100 cc. of blood plasma.)

Date	Number	Diagnosis	Glucose	Chlorine	Non-protein nitrogen	Uric acid	Creatinine	Cholesterol	Albumin	Globulin	Fibrin
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*gm.*	*gm.*	*gm.*
October 25	1		66.6	386	23.12		1.43	133.2	3.49		
November 7	2	Normal	62.4	368	20.69		1.36	144.3	3.49	2.36	0.274
November 7	3		71.6	388	22.22		1.30	88.2	3.34	2.82	0.226
November 7	4		70.8	386	17.65		1.25	100.9	3.72	3.96	0.251
October 31	5		71.2	338	52.14		2.34	73.2	2 33	4.64	0.411
November 6	5		62.5	356	26.50		1.36	82.5	2.26	5.14	0.411
November 30	5		69.0	334	30.04	1.87	1.90	83.2	2.56	6.56	0.377
October 31	6		64.3	358	20.70			89.0	2.84	3.70	0.452
November 10	6		57.1	346	28.90		1 20	88.2	3.07	3.46	0.502
November 17	6		55.3	326	33.40		1.16	100.2	3.34	3.18	0.411
November 30	6		55.3	364	20.72	1.82	1.25	125.0	3.72	4.30	0.361
October 31	7		59.0	334	19.20		1.25	78.2	2.40	3.82	0.502
November 10	7		57.1	346	25.00		1.02	75.0	2.40	3.22	0.411
November 17	7		54.0	388	30.00		1.16	94.6	3.27	3.74	0.452
November 7	8	Pneumonia	57.5	364	21.43		1.20	150.0	2.40	3.22	0.452
November 10	8		62.5	344	24.00		0.94	150.0	2.40	4.16	0.502
November 17	8		64.3	359	26.65		1.14	150.0	3.18	4.18	0.479
October 17	9		58.4	353	28.56		1.20	136.0	3.36	4.52	0.476
October 30	9		54.1	382	17.15	1.58	1.20	107.0	3.72	4.04	0.452
October 25	10		83.4	388	24.00		1.36		2.33		
November 17	11		57.1	365	30.25		1.25	88.8	3.65	3.26	0.502
November 17	12		58.4	350	20.06		1.07	115.3	3.49	3.42	0.565

The clinical data concerning the eight pneumonia cows are summarized in table 1.

A bacteriological examination of the blood and urine of cow 5 was made by Dr. F. S. Jones of this department. The blood culture was negative. He found in the urine albumin, leucocytes, epithelial cells and bacilli in large numbers. Cow 8 was slaughtered and autopsied by Dr. Jones and the junior author. The autopsy showed pleuritis and diffuse chronic pneumonia. The presence of *Bacillus bovisepticus* was established by direct culture of bits of lung tissue.

RESULTS

The results are summarized in table 2.

As can be seen from the table, cases 5, 6, 7, 8, and 9 show deviation from normal in the amount of chlorine, non-protein nitrogen, and protein fractions. The globulin is high. Cow 5 shows on October 31 a high non-protein nitrogen and creatinine. In cow 6 the cholesterol went up. In cows 11 and 12 (mild cases) the globulin was normal. In pneumonia the fibrin also is usually increased.

DISCUSSION

Chlorine. Pneumonia in the cow is evidently associated with a drop in the plasma chlorine, as it is in human beings. The rise in the plasma chlorine seems to be associated with the process of recovery. In cow 8 there was no complete recovery and the chlorine remained below normal. Cow 9 recovered completely and the chlorine reached a normal level also. Cow 5 did not recover up to November 30 and her plasma chlorine was low. Cow 6 recovered. Her chlorine content was, on November 30, normal also. The data reported by Winterstein (7) for normal cattle serum are 369.8 mgm. per cent.

Non-protein nitrogen. This blood constituent is higher when the process in the lungs is not completely healed, and reaches a normal level during recovery. The high non-protein nitrogen in cow 5 (on October 31) is a symptom of a secondary involvement of the kidneys, as confirmed by the analysis of the urine. The high creatinine content of the blood plasma of the same cow on October 31 is an additional diagnostic sign. Winterstein (7) reports for cattle 14 mgm. per cent as normal figures for non-protein nitrogen of blood serum and 1.62 for creatinine. Scheunert and Pelchrzim's (6) figures are

23.8 to 39.4 mgm. per cent for non-protein nitrogen and 1.5 to 1.8 mgm. per cent for creatinine in normal cattle.

Plasma proteins. The high globulin is evident in the presented cases 5, 6, 7, 8, and 9 of pneumonia cows. It is not possible to say definitely what caused this rise in the early stage of the disease—the process in the lungs or a certain stage of starvation due to diminshed appetite. Keese (3) showed that in horses starvation of 16 to 45 hours causes a considerable rise in the globulin fraction of the blood serum, the latter being sometimes 80 per cent of the total protein content of the serum, while in normal horse serum the globulin fraction is only 50 per cent of the total serum proteins. As our pneumonia cows showed a normal appetite during the time when the temperature went down, it is evident that the high blood serum globulin, found at this time, was due to pneumonia.

A calculation of the relative proportions of globulin nitrogen and albumin nitrogen to the total nitrogen, performed by Howe (2), gives for virgin heifers 49 per cent of the total serum protein as globulin and 51 per cent as albumin; for the pregnant heifers Howe's figures are 51 per cent of total globulin nitrogen and 49 per cent of albumin nitrogen. Our figures for serum globulin are seen to rise much above our normal figures and also those of Howe.

SUMMARY AND CONCLUSIONS

1. Pneumonia in the cow causes a drop in the blood plasma chlorine and a rise in the globulin and fibrin fractions. High non-protein nitrogen and creatinine is a sympton of secondary kidney involvement.

2. In pneumonia cows chemical analysis of blood furnishes valuable data for diagnostic and prognostic purposes.

BIBLIOGRAPHY

1. Berger, W., and Petschacher, L., Zeit. ges. exp. Med., 1923, xxxvi, 258. Vergleichende Untersuchungen zur Micro-Eiweissanalyse des Blutserums.
2. Howe, P. E., J. Biol. Chem., 1922, liii, 479. The Relation between Age and the Concentrations of Protein Fractions in the Blood of the Calf and Cow.
3. Keese, H., Biochem. Zeit., 1926, clxxviii, 184. Die Schwankungsbreite der durch verschiedene chemisch-physikalische Untersuchungsmethoden erfas-

sbaren Eigenschaften des Blutes des Pferdes unter physiologischen und pathologischen Bedingungen.

4. Myers, V. C., Practical Chemical Analysis of Blood. St. Louis, 1924, p. 22.
5. McIntosh, J. F., and Reimann, H. A., J. Clin. Invest., 1926, iii, 123. Kidney Function in Pneumonia.
6. Scheunert, A., and v. Pelchrzim, H., Biochem. Zeit., 1923, cxxxix, 17. Ueber den Gehalt des Blutes verschiedener Tierarten an Zucker, Rest-N, Harnstoff-N, Kreatininkörpern und Harnsäure nach den Folinschen Methoden.
7. Winterstein, H., Handbuch der vergleichenden Physiologie. Jena, 1925, Bd. I, S. 1118.
8. Wu, H., and Ling, S., Chinese J. Physiol., 1927, i, 161. Colorimetric Determinations of Proteins in Plasma, Cerebrospinal Fluid and Urine.

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THE IONIC ACTIVITY OF GELATIN.

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I.

INTRODUCTION.

In two other papers[1] it was shown that the monoions of weak acids in dilute solution obey the limiting Debye-Hückel equation:

$$-\log f = a\,\nu^2\sqrt{\mu} = a\,\nu^2\sqrt{\frac{1}{2}\,i\,\nu^2} \tag{1}$$

while the polyvalent ions, in the presence of NaCl, obey a modified equation:

$$-\log f = a\,\nu'\sqrt{\frac{1}{2}\,i\,\nu'} \tag{2a}$$

where

$$\nu' = \nu^2 - (\nu^2 - \nu)\frac{r_x}{18} \tag{2b}$$

where r_x is the distance in Ångstrom units between like charges in a polyvalent ion. However, these polyvalent ions show a large deviation in one direction with Mg^{++} ions and a small deviation in the opposite direction with $SO_4^{=}$ ions. Furthermore, the ions of ampholytes obey the above equations in the presence of NaCl but are anomalous with $MgCl_2$. On the other hand, the cations of amines or ampholytes do not obey the Debye-Hückel law either with NaCl or $MgCl_2$.

[1] Simms, H. S., *J. Phys. Chem.*, 1928, pending publication.

The fact that anions of ampholytes obey the modified Debye-Hückel equation ($2a$ and b) gives us a means for studying a protein. Gelatin was chosen for the purpose.

In the study of simple substances we plotted the square root of the ionic strength ($\sqrt{\mu}$) against values of the uncorrected dissociation indices (pK′ values) for each index of the substance. The slopes of these curves equal Sa, where

$$\pm S = \nu^2 - (\nu - 1)^2 = (2\nu - 1) - (\nu - 1)\frac{r_x}{9} \tag{3}$$

where ν is the valence of the ion produced by a given step in ionization, and $\pm S$ has the sign of the valence. In other words, for a given dissociation index:

$$\mathrm{pK'} - \mathrm{pK} = S\,a\,\sqrt{\mu} \tag{4}$$

where S has the value given by equation (3).

II.

Ionic Activity of Proteins.[2]

The above method cannot be applied to proteins since we cannot obtain pK′ values with any accuracy. However, we may use the following method: Let us consider two solutions of a weak electrolyte in equal concentrations and having the same equivalents of base (b') but differing in their ionic strength (due to the addition of salt to one of them). The difference (ΔpH) between the hydrogen indices of these two solutions will be the same as the difference (ΔpK′) between the uncorrected dissociation indices and will equal $\Delta(-\log f)$, hence:

$$\Delta\mathrm{pH} = \Delta\mathrm{pK} = \Delta(-\log f) = S' \cdot a \cdot \Delta\sqrt{\mu} \tag{5}$$

or

$$S' = \frac{2\,\Delta\mathrm{pH}}{\Delta\sqrt{\mu}} \tag{6}$$

[2] Attempts to determine or correct for the activity of proteins have been made by others, for example, Cohn, *Physiol. Rev.*, 1925, v, 349. Sørensen, Lang, and Lund, *J. Gen. Physiol.*, 1925–28, viii, 543. Stadie and Hawes, *J. Biol. Chem.*, 1927, lxxiv, p. xxxi.

This S' is the correct value of S only if b' is the same in both solutions. If it is different (as will be the case at high or low pH) we must write:

$$S = \frac{\Delta\text{pH} - \Delta b'/\beta'}{a \cdot \Delta\sqrt{\mu}} = S' - \frac{2\,\Delta b'}{\beta' \cdot \Delta\sqrt{\mu}} \qquad (7)$$

where $\beta' = \dfrac{\Delta b'}{\Delta\text{pH}}$ is a modification of Van Slyke's "buffer value"[3] and represents the equivalents of base required to produce unit

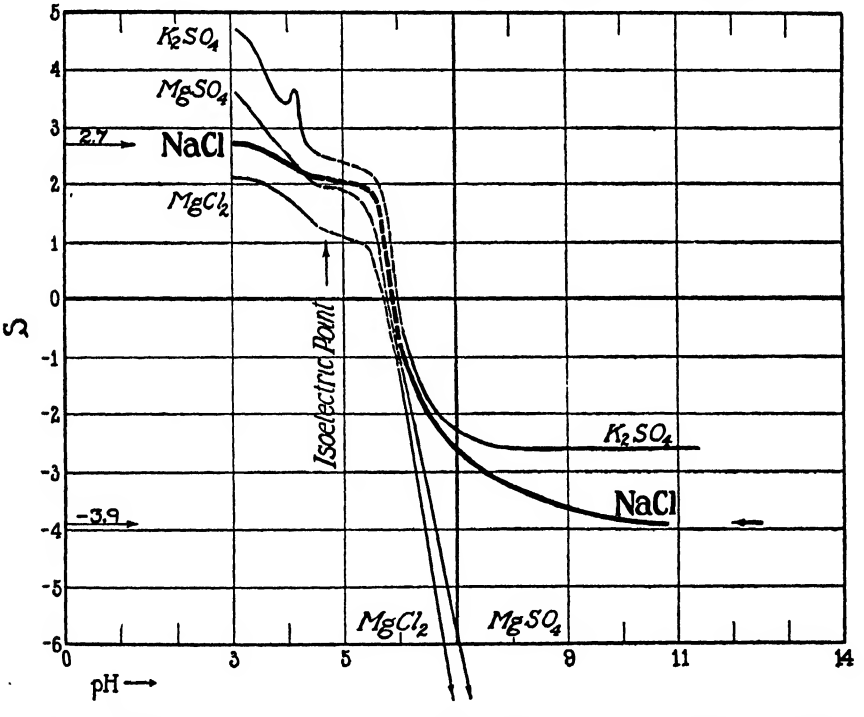

FIG. 1. Values of $S = \nu_x^s - (\nu_x - 1)^s$ for gelatin in the presence of 0.0375μ of salts. Slight experimental errors produce large differences in values of S. Each curve represents the mean of a large number of points.

changes in pH; $\Delta\text{pH} = \text{pH}_s - \text{pH}_o$; $\Delta b' = b'_s - b'_o$; and $\Delta\sqrt{\mu} = \sqrt{\mu_s} - \sqrt{\mu_o}$. The subscript s refers to solutions with salt and o without salt. S is less than S' when $\Delta b'$ is positive (algebraically).

[3] Van Slyke, *J. Biol. Chem.*, 1922, lii, 525, and Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1249. The β' values used in Table IV are those of the gelatin titration curve without salt.

III.

Results of Study of Gelatin.

We have obtained data on gelatin from pH 3 to 11. At numerous values of $\frac{b-a}{c}$ (equivalents of base) we obtained the pH of solutions without salt and at two different concentrations each of NaCl, $MgCl_2$, K_2SO_4, and $MgSO_4$. The values of ΔpH and of[4] $\Delta\sqrt{\mu}$ were used in equation (6) to calculate S'. The values of S were then calculated from equation (7) and are presented in Table IV and plotted in Fig. 1 for the dilute solutions ($0.0375\,\mu$) of salts. The curves for concentrated solutions ($0.075\,\mu$) are essentially the same but show slight deviations due to the higher ionic strength.

Previous data showed that in the presence of NaCl, anions of weak electrolytes behave normally (and cations show small deviations) while the presence of Mg^{++} ions or $SO_4^{=}$ ions causes large deviations.

[4] The values of $\sqrt{\mu}$ were obtained as follows: The *total* ionic strength is:

$$\mu = \mu_s + \mu_o \tag{8}$$

where μ_s is the ionic strength due to added salt (0.0375 and 0.0750, respectively in the two concentrations) and μ_o is the ionic strength of the solution having no added salt. The latter is calculated as follows: If the "apparent valence" of protein is unity, we may write:

$$\mu_o = \pm b'c + h + oh \tag{9}$$

(where $\pm b'c$ is always a positive number), but with an "apparent valence" of ν_A we get

$$\mu_o = \pm \frac{\nu_A^2 + 1}{2} b'c + h + oh \tag{10}$$

Our preliminary estimation of S_M was 2.0 in acid solution and 3.0 in alkaline solution. Equation (15) gives values of ν_A equal to 1.5 and 2.0. These were used in equations (8) and (10) to obtain the μ values used in Table IV. The final values of S_M are 2.7 and 3.9 (corresponding to ν_A equals 1.85 and 2.45). Although we would expect the latter values would be more correct, they appear to be too high. As an approximation we have used the same value (1.5) of ν_A at all points in the acid titration and the same value (2.5) at all points in alkali titration and we have neglected h and oh in equation (10).

TABLE I.

Deviations (ΔS) Produced in Weak Electrolytes by 0.0375μ of Salts.

Substance	Distance r_z	Valence ν	Apparent valence ν_A	ΔS in acid titration $MgCl_2$	ΔS in alkaline titration		
					$MgCl_2$	$MgSO_4$	K_2SO_4
	Å						
Citric acid.....	7.4	2	1.7		−9		
Citric acid.....	7.4	3	2.2		−29		
Oxalic acid.....	4.5	2	1.8		−17		
Malonic acid.....	5.7	2	1.7		−8.3	−5.0	+0.7
Succinic acid.....	7.0	2	1.6		−1.3		
Azelaic acid.....	12.1	2	1.3		−0.8		
Aspartic acid.....	7.0	2	1.6		−9.5		
Aspartic acid.....		1	1	−0.2			
(Glycine).....		1	1	−0.2	−6		
(Aminoethanol).....		1	1	−0.8			

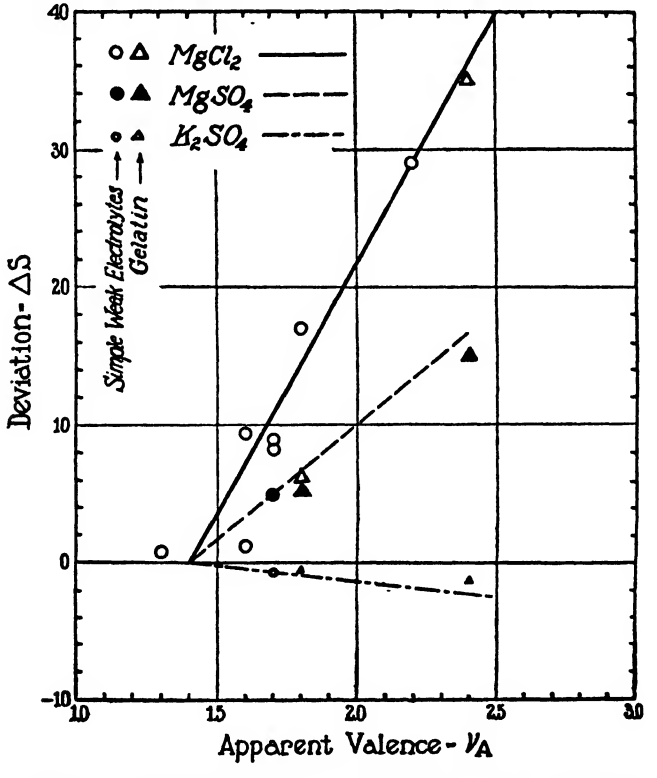

FIG. 2. Relation between "apparent valence" and the deviation produced by 0.0375μ of various salts on simple weak electrolytes and on gelatin. The lines correspond to equations (12*a*), (12*b*), and (12*c*).

Hence we will take the NaCl data as "normal" and note the deviations caused by other ions. This deviation will be:

$$\Delta S = S_o - S_x \tag{11}$$

where S_o is the value of S with NaCl, and S_x is the value of S with any other salt.

The values of ΔS for some ions of simple weak electrolytes are given in Table I and are plotted in Fig. 2. It will be seen that the data with $MgCl_2$ fall roughly on the empirical curve with the equation

$$\nu_A = 1.4 - \frac{\Delta S}{25} \tag{12a}$$

If we assume the same origin for the curves of $MgSO_4$ and K_2SO_4, and use the data with malonic acid to determine the slope; we get, for $MgSO_4$

$$\nu_A = 1.4 - \frac{\Delta S}{17} \tag{12b}$$

For K_2SO_4:

$$\nu_A = 1.4 + \frac{\Delta S}{2.3} \tag{12c}$$

These equations apply only to the effect of $0.0375\,\mu$ of these salts on polyanions.

The effect of these salts on gelatin is given in Table II where the values found in alkaline titration (Columns 7 to 9) are seen to agree with those calculated by the above formulas (Columns 10 to 12). These points for gelatin are plotted in Fig. 2 (triangles) and are seen to agree with the curves found for the simple weak electrolytes.

In acid solution gelatin behaves like aspartic acid or glycine with $MgCl_2$. Unfortunately we have no data on polycations with which to make quantitative comparison in acid solution. The qualitative behavior agrees with our expectations.

On the basis of these observations there is every reason to believe that gelatin in dilute solution (2.5 per cent or less) behaves like a polyvalent ampholyte with distant ionizable groups[5] and that the

[5] The nature and sources of these groups will be discussed in the following paper.

ionization is purely that of a weak electrolyte showing the same effects in the presence of Mg^{++} or $SO_4^{=}$ ions that are shown by the simple weak electrolytes.

These deviations are similar to the combination of proteins with inorganic ions as observed by other methods[6] and the combination of simpler substances with inorganic ions.[7]

TABLE II.

Deviations (ΔS) Produced in Gelatin by 0.075μ of Salts (Distance r_x >18 Å.u.)

pH	Valence ν	Apparent valence ν_A	ΔS values								
			Found in acid titration			Found in alkaline titration			Calculated for alkaline titration		
			$MgCl_2$	$MgSO_4$	K_2SO_4	$MgCl_2$	$MgSO_4$	K_2SO_4	$MgCl_2$	$MgSO_4$	K_2SO_4
3.4	57	1.8	−0.6	+0.2	+1.1						
7.1	16	1.8				−6.3	−5.3	+0.5	−15	−6.5	+0.9
11.0	43	2.4				−30 to −40*	−15*	+1.3	−36	−17	+2.3

* The values for $MgCl_2$ (−30 to −40) and $MgSO_4$ (−15) at pH 11 were estimated by extrapolation.

IV.

Relation between Activity, Valence, and Distance.

Assuming a given molecular weight we may calculate the corresponding number of acid or basic groups from the base or acid "combining capacity." This calculated valence (ν_x) will be a large number

[6] Northrop and Kunitz, *J. Gen. Physiol.*, 1925–26, ix, 351 and unpublished data. Hastings and Sendroy, *J. Biol. Chem.*, 1927, lxxi, 723. Loeb, R. F., and Nichols, *J. Biol. Chem.*, 1927, lxxiv, 645. Adair, *J. Biol. Chem.*, 1925, lxiii, 517, 529. Austin, Sunderman, and Camack, *J. Biol. Chem.*, 1926, lxx, 427.

[7] Pfeiffer and collaborators, *Z. physik. Chem.*, 1924, cxxxiii–cxliii.

In this connection we will mention that a quantitative measurement of the interaction of glycine and phosphoric diion gives the mass action equation:

$$k = \frac{[\text{glycine}] \times [HPO_4^{=}]}{[\text{Combined}]} = 0.040$$

as shown by the effect of glycine on pK_2' of H_3PO_4. The data will be published later. Similar, but more complex, relations have been found to apply to the effects of Mg^{++} and of $SO_4^{=}$ on ions of oxalic acid.

which if used in the Debye-Hückel equation (1) will give impossible values for the effect of protein on ionic strength. This is because equation (1) assumes the charges to be located at a single point.

A polyvalent ion with charges very far apart would behave like a number of univalent ions. Equation (2) is derived to allow for finite distances between like charges. The mean distance r_x may be calculated from the equation (derived from equation (3)):

$$r_x = \frac{9(2\nu - S - 1)}{\nu - 1} \tag{13}$$

From the base-combining capacity (1.75 equivalents per 2500 gm.) and the acid-combining capacity (2.30), we may calculate the maxi-

TABLE III.

Assumed molecular weight	ν_x No. of acid groups	ν_x No. of basic groups	Mean distance r_x (in Å.u.)	
			Between acid groups ($-S_M = -3.9$)	Between basic groups ($S_M = 2.7$)
96,000	67	88	17.6	17.8
61,500*	43	57	17.4	17.7
30,000	21	27.5	16.7	17.4
20,000	14	18.4	16.0	17.1

*See Foot-note 8.

mum valence ν_M of acid and basic groups corresponding to various assumed molecular weights.

The NaCl curve in Fig. 1 reached a maximum (S_M) of + 2.7 and a minimum ($-S_M$) of −3.9 (in acid and alkaline solutions, respectively). With these values of ν_M and S_M we may calculate the mean distance r_x from equation (13). These are given in Table III for various assumed molecular weights.[8]

[8] The value 96,000 was obtained by Smith, *J. Am. Chem. Soc.*, 1921, xliii, 1350. The more accurate value of 61,500 (at 25°C.) has since been obtained by Kunitz, *J. Gen. Physiol.*, 1926–27, x, 811. The arbitrary values 30,000 and 20,000 are ncluded in Table III to show that the molecular weight has little effect when the valence is above 10.

Since the formula probably gives low values for long distances, we conclude that the distances are *18Å. u. or over.*

The *probable* distance may be estimated if we consider that the *minimum* distance between acid groups in a protein molecule is about that in aspartyl-aspartic acid anhydride, which is 10 Å.u. Only 1 amino acid molecule in 14 in gelatin is a dicarboxylic acid. Hence the probable mean distance is 30 to 60 Å.u.; but since some will be closer than others the *effective* mean distance will be less, probably between 18 and 25 Å.u.

Another check on this distance is found in the "titration index dispersion."[9] That calculated for 18 Å.u. or over, is 0.7 or less. This agrees with the titration curve which has a dispersion of not more than 0.7, and apparently much less.

V.

The Gelatin Molecule.

The data indicate that the gelatin molecule is large; that the dielectric constant of the medium between these groups is not greatly different from that of water;[10] and that the free ionizable groups are all functioning and are accessible to the inorganic ions in solution. We conclude that the protein molecule is spongy or arborescent in shape with molecules of solvent and of other solutes invading the interstices. It is reasonable to suppose, furthermore, that the shape and size of the molecule changes with pH since the like charges will repel each other[10] and the increase in ionic strength may also influence the

[9] The term "titration index dispersion" is used to refer to the difference between two "titration indices" having the same intrinsic indices. Thus since oxalic acid is symmetrical both groups have the same intrinsic indices (2.55); but its "titration index dispersion" ($pG_2 - pG_1$) is 2.93. In the case of sebacic acid $pG_2 - pG_1 = 0.88$. These values depend upon the distances r_d (4.5 and 14.3, respectively) and the distances r_m (1.2 and 4, respectively) between the charges in the diion and the monoion.

A divalent acid with $r_d = 18$ and $r_m = 5$ would have a dissociation index dispersion of $\Delta pK = 0.85$ or a *titration index dispersion of 0.7*, while a greater distance would give a smaller dispersion.

[10] Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1251.

shape.[11] Such a change in shape and size would be roughly analogous to the opening and closing of a flower.

VI.

The Apparent Valence of Gelatin.

If we assume a point charge (an impossible condition) r_z in equation (3) will equal zero and we get:

$$\pm S = 2\,\nu_A - 1 \tag{14}$$

or

$$\nu_A = \frac{\pm S + 1}{2} \tag{15}$$

where ν_A is the "apparent valence" under the assumption of a point charge ($\pm S$ is always a positive number). If we use the maximum ($S_M = 2.7$) and minimum ($-S_M = -3.9$) values of S obtained from Fig. 1 we find that the "maximum apparent valence" is $\nu_A = 2.4$ for acid groups (in alkaline solution) and $\nu_A = 1.8$ for basic groups (in acid solution). These apparent valences have no physical significance but we may substitute $\nu_A{}^2$ in the unmodified equation (1) to obtain the *maximum* effect of gelatin on the ionic strength.[4] The *true* effect appears to be even lower than the above values would indicate.

VII.

EXPERIMENTAL.

The gelatin used was ash-free isoelectric gelatin kindly furnished by Dr. John H. Northrop.

Most of the data were obtained on solutions which were 0.005 M per 2500 gm. (an arbitrary molecular weight), namely 1.25 per cent gelatin. The solutions at high and low pH were twice as strong

[11] The last two conclusions are contrary to Svedberg and Nichols (Svedberg and Nichols, *J. Am. Chem. Soc.*, 1927, xlix, 2920) whose primary assumptions were that the hemoglobin molecule is spherical and its size is independent of pH. See also Ghosh, *J. Chem. Soc.*, 1928, cxxxiii, 117.

TABLE IV.

Titration Data of Gelatin without Salt and in the Presence of 0.0375μ (D.—) and and 0.0750μ (C.—) of Salts.

In order to condense this table we give only about half the experimental data used in Fig. 1.

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	1.808	−4.000*			
None	2.133	−3.000*			
None	2.448	−2.500*			
None	2.894	−2.000*			
None	3.010	−2.000	0.85		
D.—$MgCl_2$	3.080	"		1.4	2.0
C.—$MgCl_2$	3.113	"		1.2	1.7
D.—NaCl	3.109	"		1.8	2.6
C.—NaCl	3.133	"		1.4	1.9
D.—$MgSO_4$	3.140	"		2.4	3.5
C.—$MgSO_4$	3.196	"		2.1	3.0
D.—K_2SO_4	3.189	"		3.3	4.7
C.—K_2SO_4	3.256	"		2.8	3.9
None	3.074	−1.900	1.06		
D.—$MgCl_2$	3.167	"		1.7	2.2
C.—$MgCl_2$	3.199	"		1.4	1.8
D.—NaCl	3.194	"		2.2	2.9
C.—NaCl	3.216	"		1.6	2.0
D.—$MgSO_4$	3.223	"		2.8	3.6
C.—$MgSO_4$	3.275	"		2.3	2.9
D.—K_2SO_4	3.270	"		3.6	4.7
C.—K_2SO_4	3.329	"		2.9	3.7
None	3.245	−1.700	1.17		
D.—$MgCl_2$	3.328	"		1.5	1.8
C.—$MgCl_2$	3.370	"		1.4	1.6
D.—NaCl	3.362	"		2.1	2.5
C.—NaCl	3.390	"		1.6	1.9
D.—$MgSO_4$	3.394	"		2.7	3.2
C.—$MgSO_4$	3.431	"		2.0	2.4
D.—K_2SO_4	3.438	"		3.5	4.2
C.—K_2SO_4	3.497	"		2.8	3.3
None	3.391	−1.500	0.92		
D.—$MgCl_2$	3.488	"		1.7	2.0
C.—$MgCl_2$	3.534	"		1.5	1.7
D.—NaCl	3.522	"		2.3	2.7
C.—NaCl	3.553	"		1.7	2.0
D.—$MgSO_4$	3.542	"		2.6	3.0
C.—$MgSO_4$	3.586	"		2.1	2.4
D.—K_2SO_4	3.588	"		3.5	4.0
C.—K_2SO_4	3.649	"		2.8	3.2

* The asterisk on values of $(b - a)/c$ indicate that those solutions were 0.01 M per 2500 gm. (*i.e.*, 2.50 per cent). All other solutions were 0.005 M per 2500 gm. (*i.e.*, 1.25 per cent).

TABLE IV—*Continued.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	3.573	−1.300	1.12		
D.—$MgCl_2$	3.671	"		1.7	1.8
C.—$MgCl_2$	3.705	"		1.4	1.5
D.—NaCl	3.706	"		2.3	2.5
C.—NaCl	3.742	"		1.8	1.9
D.—$MgSO_4$	3.720	"		2.5	2.7
C.—$MgSO_4$	3.756	"		1.9	2.1
D.—K_2SO_4	3.764	"		3.3	3.6
C.—K_2SO_4	3.813	"		2.5	2.7
None	3.742	−1.100	1.4		
D.—$MgCl_2$	3.842	"		1.6	1.8
C.—$MgCl_2$	3.871	"		1.3	1.4
D.—NaCl	3.876	"		2.2	2.4
C.—NaCl	3.911	"		1.7	1.8
D.—$MgSO_4$	3.882	"		2.3	2.5
C.—$MgSO_4$	3.916	"		1.8	2.0
D.—K_2SO_4	3.926	"		3.0	3.3
C.—K_2SO_4	3.971	"		2.1	2.4
None	3.791	−1.000	1.4		
D.—$MgCl_2$	3.898	"		1.7	1.8
C.—$MgCl_2$	3.930	"		1.4	1.5
D.—NaCl	3.930	"		2.2	2.3
C.—NaCl	3.969	"		1.8	1.9
D.—$MgSO_4$	3.947	"		2.5	2.6
C.—$MgSO_4$	3.976	"		1.9	2.0
D.—K_2SO_4	3.987	"		3.2	3.3
C.—K_2SO_4	4.028	"		2.4	2.5
None	3.973	−0.800	1.5		
D.—$MgCl_2$	4.077	"		1.6	1.6
C.—$MgCl_2$	4.099	"		1.2	1.2
D.—NaCl	4.113	"		2.2	2.3
C.—NaCl	4.138	"		1.6	1.6
D.—$MgSO_4$	4.116	"		2.2	2.3
C.—$MgSO_4$	4.141	"		1.6	1.6
D.—K_2SO_4	4.160	"		4.4	4.5
C.—K_2SO_4	4.190	"		3.1	3.2
None	4.14	−0.600	1.0		
D.—$MgCl_2$	4.256	"		1.6	1.6
C.—$MgCl_2$	4.267	"		1.7	1.7

TABLE IV—*Continued.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
D.—NaCl	4.295	−0.600		2.2	2.3
C.—NaCl	4.304	"		1.5	1.5
D.—$MgSO_4$	4.29	"		2.2	2.4
C.—$MgSO_4$	4.30	"		1.5	1.5
D.—K_2SO_4	4.328	"		2.6	2.7
C.—K_2SO_4	4.349	"		1.9	1.9
None	4.41	−0.300	1.1		
D.—$MgCl_2$	4.512	"		1.3	1.3
C.—$MgCl_2$	4.503	"		0.8	0.8
D.—NaCl	4.554	"		1.9	1.9
C.—NaCl	4.566	"		1.4	1.4
D.—$MgSO_4$	4.556	"		2.0	2.0
C.—$MgSO_4$	4.542	"		1.1	1.1
D.—K_2SO_4	4.596	"		2.5	2.5
C.—K_2SO_4	4.607	"		1.8	1.8
None	4.7	0	1.0		
D.—$MgCl_2$	4.847	"		>1	>1
C.—$MgCl_2$	4.827	"		<1	<1
D.—NaCl	4.891	"		>2	>2
C.—NaCl	4.881	"		<2	<2
D.—$MgSO_4$	4.855	"		>2	>2
C.—$MgSO_4$	4.859	"		<2	<2
D.—K_2SO_4	4.921	"		>2	>2
C.—K_2SO_4	4.898	"		<2	<2
None	5.1	0.300	0.4		
D.—$MgCl_2$	5.28	"		(2.7)	(2.7)
C.—$MgCl_2$	5.23	"		(1.7)	(1.7)
D.—NaCl	5.38	"		(4.0)	(4.0)
C.—NaCl	5.31	"		(1.7)	(1.7)
D.—$MgSO_4$	5.30	"		(2.7)	(2.7)
C.—$MgSO_4$	5.26	"		(1.7)	(1.7)
D.—K_2SO_4	5.38	"		(4.0)	(4.0)
C.—K_2SO_4	5.36	"		(2.5)	(2.5)
None	6.10	0.500	0.17		
D.—$MgCl_2$	6.019	"		−1.2	−1.2
C.—$MgCl_2$	5.960	"		−1.4	−1.4
D.—NaCl	6.055	"		−0.8	−0.8
C.—NaCl	5.977	"		−1.2	−1.2
D.—$MgSO_4$	6.033	"		−1.1	−1.1
C.—$MgSO_4$	6.004	"		−1.0	−1.0
D.—K_2SO_4	6.07	"		−0.5	−0.5
C.—K_2SO_4	6.02	"		−0.8	−0.8

TABLE IV—*Continued.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	6.82	0.600	0.12		
D.—$MgCl_2$	6.61	"		−3.3	−3.3
C.—$MgCl_2$	6.51	"		−3.1	−3.1
D.—NaCl	6.68	"		−2.2	−2.2
C.—NaCl	6.63	"		−1.9	−1.9
D.—$MgSO_4$	6.48	"		−5.4	−5.4
C.—$MgSO_4$	6.65	"		−1.7	−1.7
D.—K_2SO_4	6.65	"		−2.7	−2.7
C.—K_2SO_4	6.45	"		−3.7	−3.7
None	7.72	0.700	0.11		
D.—$MgCl_2$	7.17	"		−9.1	−9.1
C.—$MgCl_2$	7.49	"		−2.3	−2.3
D.—NaCl	7.54	"		−3.0	−3.0
C.—NaCl	7.56	"		−1.6	−1.6
D.—$MgSO_4$	7.53	"		−3.1	−3.1
C.—$MgSO_4$	7.65	"		−0.6	−0.6
D.—K_2SO_4	7.58	"		−2.3	−2.3
C.—K_2SO_4	7.61	"		−1.1	−1.1
None	8.66	0.800	0.12		
D.—NaCl	8.46	"		−3.4	−3.4
C.—NaCl	8.48	"		−1.9	−1.9
D.—K_2SO_4	8.49	"		−2.9	−2.9
C.—K_2SO_4	8.56	"		−1.0	−1.0
None	9.414	0.900	0.19		
D.—NaCl	9.209	"		−3.6	−3.8
C.—NaCl	9.214	"		−2.1	−2.2
D.—K_2SO_4	9.297	"		−2.0	−2.1
C.—K_2SO_4	9.235	"		−1.9	−2.0
None	9.792	1.000	0.26		
D.—NaCl	9.592	"		−3.6	−3.9
C.—NaCl	9.580	"		−2.3	−2.5
D.—K_2SO_4	9.646	"		−2.6	−2.9
C.—K_2SO_4	9.629	"		−1.8	−2.0
None	10.408	1.300	0.57		
D.—NaCl	10.254	"		−2.9	−3.4
C.—NaCl	10.237	"		−2.0	−2.4
D.—K_2SO_4	10.303	"		−2.0	−2.4
C.—K_2SO_4	10.266	"		−1.6	−1.9

TABLE IV—*Concluded.*

Salt	pH	$\frac{b-a}{c}$	Mean β'	S'	S
None	10.573	1.400	0.40		
D.—NaCl	10.434	"		−2.6	−4.3
C.—NaCl	10.410	"		−1.9	−3.0
D.—K_2SO_4	10.476	"		−1.8	−3.1
C.—K_2SO_4	10.456	"		−1.3	−2.1
None	11.442	2.000*	0.18		
D.—NaCl	11.390	"		−1.3	(−6)
C.—NaCl	11.307	"		−1.1	(−4)
D.—K_2SO_4	11.410	"		−0.8	−2.6
C.—K_2SO_4	11.384	"		−0.8	−2.8

(0.01 M or 2.5 per cent). At each equivalent of base $(b - a)/c$ which was studied, two solutions were made up without salt, also one "dilute" (0.0375μ) and one "concentrated" (0.0750μ) of each of the salts: NaCl, $MgCl_2$, K_2SO_4, and $MgSO_4$. Each solution contained 5 cc. of a mother solution of gelatin (of twice the concentration) and was made up to 10 cc. All readings at a given value of $\frac{b-a}{c}$ were taken within a few minutes of each other to reduce experimental error in the relative values to a minimum. One solution without salt was measured first and the other last, those with salt coming in between. The same two bubbling water-jacketed hydrogen electrode cells[12] were used in all measurements. Saturated KCl junction was assumed constant. The pH standard was 0.100 M HCl equals pH 1.075 at 25°C. The data are given in Table IV. The two values without salt are averaged in each case where there was any difference.

The values of b' used in equation (7) to calculate values of S' and S were calculated by the formula as described previously.[13]

$$b' = \frac{b-a}{c} + h - \frac{oh}{c}$$

[12] Simms, *J. Am. Chem. Soc.*, 1923, xlv, 2503.
[13] Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

VIII.

SUMMARY.

2.5 and 1.25 per cent gelatin have been titrated potentiometrically in the absence of salts and in the presence of two concentrations (0.0750 and 0.0375μ) of NaCl, $MgCl_2$, K_2SO_4, and $MgSO_4$. The data have been used to calculate values of $\pm S = \nu^z - (\nu - 1)^z$, where $\nu^z = \nu^2 - (\nu^2 - \nu) r_z/18$.

The maximum and minimum values of S with NaCl were used to calculate the mean distance (r_z) between like charges in gelatin. This is found to be 18 Å.u. or over (between acid or basic groups) which agrees with the probable value and the titration index dispersion. Thus the data with NaCl are shown to be normal and to obey the equation found to hold for simple weak electrolytes; namely, $pK' - pK = Sa\sqrt{\mu}$ where S is related to the valence and distance by the above equations.

Using the NaCl data as a standard the deviations (ΔS) produced by the other salts are calculated and are found to agree quantitatively with the deviations calculated from equations derived for the simple weak electrolytes. This shows that in gelatin, as in the simple electrolytes, the deviations are related to the "apparent valences" (values which are a function of the true valence and the distance between the groups).

The maximum "apparent valences" of gelatin are 2.4 for acid groups (in alkaline solution) and 1.8 for basic groups (in acid solution). These values correspond to the hypothetical condition of zero distance between the groups. They have no physical significance but have a practical utility first as mentioned above, and second in that they may be used in the unmodified Debye-Hückel equation to give the maximum effect of gelatin on the ionic strength. The true effect is probably even lower than these values would indicate.

The data indicate that gelatin is a weak polyvalent ampholyte having distant groups and that the molecule has an arborescent structure with interstices permeated by molecules of the solvent and other solutes. The size and shape probably vary with the pH.

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THE NATURE OF THE IONIZABLE GROUPS IN PROTEINS.

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I.

Dissociation Indices of Amino Acids and Peptides.

In Table I will be seen values of the dissociation indices[1] of various divalent amino acids and peptides (*i.e.*, having one free α-amino group and one free α-carboxyl group).

In Table II are the dissociation indices of some trivalent amino acids and peptides which, in addition to the α-amino group and α-carboxyl group, have a third ionizable group.

The various theories of protein structure now under discussion are all in agreement with the assumption that one amino group and one carboxyl group of each amino acid in a protein molecule are bound in some manner which prevents them from ionizing.[2]

Hence we would expect the ionizable groups[3] in the protein molecule

[1] The term "index" is used here, as previously defined, to indicate the negative logarithm of a value; *e.g.*, "dissociation index" = pK = $-\log K$, or "titration index" = pG = $-\log G$.

[2] The "chain" theory, however, assumes that in each protein molecule one α-amino group and one α-carboxyl group are free; but such an assumption is unwarranted by experimental accuracy even if the chain theory were known to be correct and the chains had no branches. In gelatin it would raise the predicted sum of groups from 4.00 to 4.08 which would be in better agreement with the experimental value of 4.05. In egg albumin it would raise the basic groups from 1.6 to 1.75 which would better agree with the experimental value of 2.1. But despite the better agreement it is preferable to simplify our assumptions and attribute the ionizable groups to only the extra groups of trivalent amino acids.

[3] There is still a school of chemists who believe that the charges on a protein molecule are not due to the ionizable groups but to its charge as a colloidal particle. In this article, however, we feel justified in making the unqualified assumption that the charges *in dilute solution* are due to free ionizable groups.

to be due to the "extra" groups in the trivalent amino acids (Table II). It does not follow, however, that the bound groups are always the α groups. For instance in lysine we can conceive of the α-carboxyl and the ϵ-amino group being bound, leaving the α-amino group free to ionize. The titration index will be different than if the α-amino group were bound and the ϵ-amino group were free. However, the data indicate that the latter structure prevails. The same is true of arginine and histidine; the α-amino groups are bound and the extra basic groups are free (in so far as they exist in that form, see below).

TABLE I.

Titration Indices of Divalent Amino Acids and Peptides (and Related Monovalent Electrolytes).

Substance	pG_1' (– COOH)	pG_2' (– NH_3)	Author*
Acetic acid	$4.740-0.9a\sqrt{\mu}$		S.
Glycollic acid	3.82		S.
Amino ethanol		$9.470 + 0.5a\sqrt{\mu}$	S.
Glycine ethyl ester hydrochloride		7.655	S.
Glycine	$2.365-0.08a\sqrt{\mu}$	$9.715-a\sqrt{\mu}$	S.
Alanine	2.35	9.72	L.S.
Sarconine	2.23	10.01	L.S.
Tryptophane	2.266	9.372	S.
Valine	2.28	9.65	L.B.
Glycyl-glycine	3.12	8.07	L.S.
Sarcosyl-glycine	3.10	8.51	L.S.
Glycyl-sarconine	2.83	8.54	L.S.
Sarcosyl-sarconine	2.86	9.10	L.S.
Alanyl-alanine	3.17	8.42	L.S.
Glycyl-valine	2.28	8.30	L.B.
Glycyl-leucine	3.18	8.29	S.
Glycyl-alanine	3.15	8.25	S.
Glycyl-asparagine	2.9	8.3	S.
Glycyl-glycyl-glycine	3.26	7.91	L.S.
Glycyl-alanyl-alanyl-glycine	3.30	7.9	L.S.
Alanyl-alanine anhydride (enol group = 13.5)			L.B.

* L.S. represents Levene and Simms; L.B., Levene and Bass; and S., Simms.

TABLE II.

Titration Indices of Trivalent Amino Acids.

Substance	pG′ $\alpha-COOH$	pG′ $\alpha-NH_2$	Third group		Approximate pG′ in protein	Author*
			Group	pG′		
Aspartic acid	$2.05-0.6a\sqrt{\mu}$	$10.00-2.3a\sqrt{\mu}$	$-COOH$	$3.87-1.2a\sqrt{\mu}$		S.
Glycyl aspartic acid	2.81	8.60	$-COOH$	4.45	3.5	S.
Aspartyl glycine	2.10	9.07	$-COOH$	4.53		L.S.
Glutamic acid	2.11	9.45	$-COOH$	4.06		L.S.
Histidine	1.46	9.41	$=NH$	6.06	6.1	S.
Arginine	2.29	9.64	$=NH$	8.15	8.1	S.
Tyrosine	2.24	10.28	$-OH$	9.21	9.4	S.
Lysine	2.04	9.06	$-NH_2$	10.45	10.6	L.S

*L.S. represents Levene and Simms; and S., Simms.

TABLE III.

Content of Amino Acids in Gelatin.

Amino acid		Weight	Molecular weight	Mols per 61,500 gm.	Mols per 2500 gm.
		per cent			
Divalent	Glycine	25.5	75	209	8.5
	Alanine	8.7	89	60	2.4
	Leucine	7.1	131	33	3.4
	Serine	0.4	105	2	0.1
	Phenylalanine	1.4	165	5	0.2
	Proline	9.5	115	51	2.1
	Oxyproline	14.1	131	66	2.7
Total divalent.....		66.7		426	17.4
Trivalent	Aspartic acid	3.4	133	16	0.64
	Glutamic acid	5.8	147	24	1.0
	Histidine	0.9	155	4	0.15
	Arginine	8.2	174	29	1.2
	Tyrosine	0.01	181	0.03	0
	Lysine	5.9	146	25	1.0
Total trivalent.....		24.2		98	4.0
Total amino acids.....		90.9		525	21.4

II.

The Predicted Ionizable Groups of Proteins.

In Table III are the percentages of various amino acids in gelatin as found by Dakin.[4] Since we are interested only in the trivalent amino acids these are listed in Table IV in the column of "predicted" equivalents (per 2500 gm., an arbitrary unit weight). The third column gives the predicted values for gelatin and the eighth column for egg albumin.

TABLE IV.

Equivalents (per 2500 gm.) of the groups found, compared with the equivalents predicted from the content of the respective amino acids. The values for gelatin must be multiplied by 24.6 to obtain the equivalents per molecular weight of 61,500 (Kunitz); and the egg albumin values should be multiplied by 13.5 for molecular weight of 33,800.

1		2	3	4	5	6	7	8	9	10
			Gelatin					Egg albumin		
Groups	Sources	Approximate indices*	Predicted	1st titration		2nd titration		Predicted	Found	Difference
				Found	Difference	Found	Difference			
Acidic	Dicarboxylic acids	$pG_1' = 3.5$*	1.65	1.75	+0.1	1.75	+0.1	?	1.6	?
	Tyrosine	$pG_5' = 9.4$	0	0	0	0	0	0.6	0.4	−0.2
Basic	**Unknown**	$pG_2' = 4.6$	0	1.0	+1.0	1.05	+1.05	0	0.9	+0.9
	Histidine	$pG_3' = 6.1$	0.15	0.2	0.05	0.15	0	0.3	0.3	0
	Arginine	$pG_4' = 8.1$	1.2	0.2	−1.0	0.2	−1.0	0.7	0.3	−0.4
	Lysine	$pG_6' = 10.6$*	1.0	0.9	−0.1	0.9	−0.1	0.6	0.6	0
Total acid groups ("base-binding capacity")			1.65	1.75	+0.1	1.75	+0.1	?	2.0	?
Total basic groups ("acid-binding capacity")			2.35	2.30	−0.05	2.30	−0.05	1.6	2.1	+0.5
Sum of **arginine** + "**4.6 group**"			1.2	1.2	0	1.25	0.05	0.7	1.2	0.5

* The titration indices (pG' values) found, were those given above except in three cases: pG_1' in egg albumin is 2.9 (instead of 3.5). pG_6' in egg albumin was 10.8, and in the first titration of gelatin was 10.4 (instead of 10.6 as found in the second titration).

[4] Dakin, *J. Biol Chem.*, 1920, xliv, 499.

In Fig. 1, Curve *B*, we see the way the titration curve of gelatin would look if these groups occurred in gelatin in the quantities "predicted" by the amino acid content. The isoelectric point would be around pH 8.0 instead of 4.7. The experimental curve (*A*) of gelatin has an isoelectric point at 4.7 and has a different shape between pH 4 and 10.

We have analyzed the experimental curve of gelatin (Curve *A*, Fig. 1) in order to see if it could be resolved into groups with the

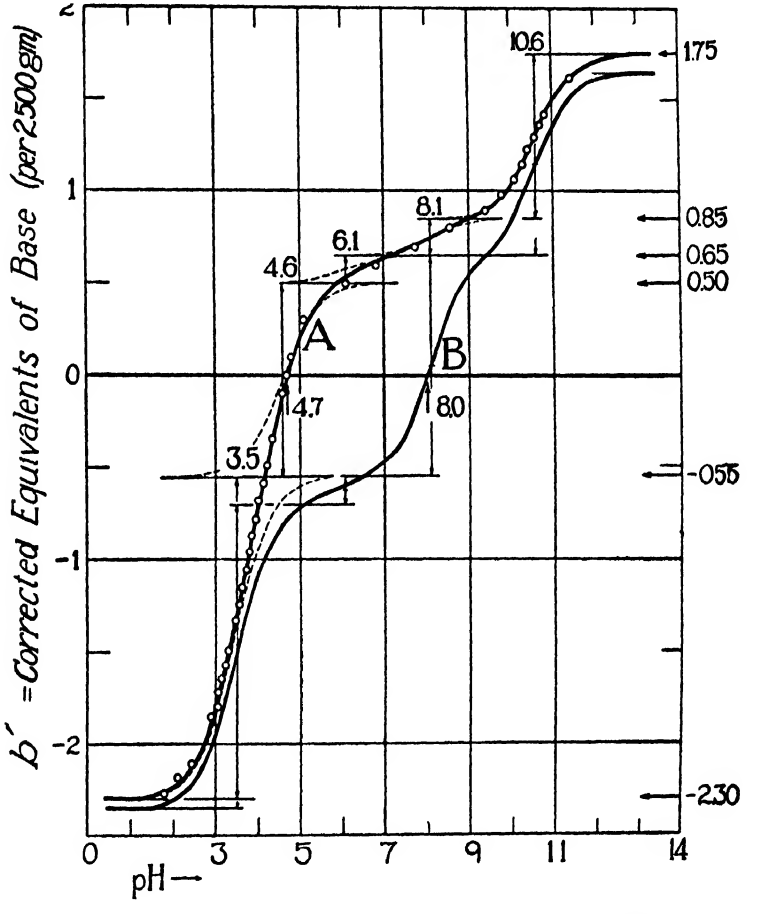

FIG. 1. Gelatin titration curves. Curve *A*. Experimental points and curve corresponding to values marked "found" in Table IV. Curve *B*. Curve "predicted" from trivalent amino acids (see Table IV).

indices given in Table IV. Our method was similar to that of E. J. Cohn[5] except that we restricted ourselves to titration indices which the free ionizable groups should have in the protein molecule, and furthermore we did not consider the isoelectric point as a transition between one ionizable group and another. It has been shown by Northrop[6] that gelatin contains at least two constituents not chemically bound. It is probable that gelatin and other proteins consist of mixtures of numerous constituents. In speaking of the "gelatin molecule" we recognize that we are dealing with a mixture of different molecules. This is kept in mind in the construction of the "predicted" curve and the analysis of the experimental curve. The isoelectric points do not represent transition points between ionic species as is the case with pure chemical substances having groups close together.

III.

The Actual Groups of Proteins.

The analysis of the experimental gelatin curve (Fig. 1, Curve *A*, and Columns 4 and 6 of Table IV) shows that the total number of acid groups agrees within 0.1 equivalent (per 2500 gm.) with the value predicted from the content of acidic amino acids.

The total number of basic groups agrees (within 0.05 equivalent) with the predicted value.[7] However, the quantity of arginine is a whole equivalent (1.0 eq.) too low in quantity. Furthermore, *the deficiency in arginine is compensated by the existence in gelatin of one equivalent of a basic group of unknown source with a titration index (pG'_s) at 4.6.*

A glance at the last three columns of Table IV shows that the same is true of egg albumin (see Fig. 2). Arginine group is 0.4 equivalent

[5] Cohn, *Physiol Rev.*, 1925, v, 349.

[6] Northrop, *J. Gen. Physiol.*, 1926–27, x, 161.

[7] This agreement is not found if we use the content of histidine found by Van Slyke (0.5) instead of the value of Dakin (0.15). The latter value agrees with the amount of histidine group (0.2) in gelatin. However, the relation between arginine and 4.6 group is not affected by the value assumed for histidine.

too low, while there is 0.9 equivalent of the "4.6 group" in egg albumin.[8]

The nature of the "4.6 group" is unknown. It is undoubtedly a basic (amino) group. The aliphatic amino groups have indices which range from 12.0 for diethyl amine to 7.65 for glycine ester hydrochloride. Unsaturated bases are weaker: guanidine has a basic group at 6.0; arginine at 8.15, and histidine at 6.06. However, bases as weak as 4.6 are not found except in the very unsaturated systems of aniline (4.6), cytosine (4.6), and isocystosine (4.0). The latter bases have an amino group attached to a conjugated unsaturated cyclic system.

Hence we are safe in concluding that a basic group with a conjugated unsaturated (and perhaps cyclic) structure occurs in gelatin

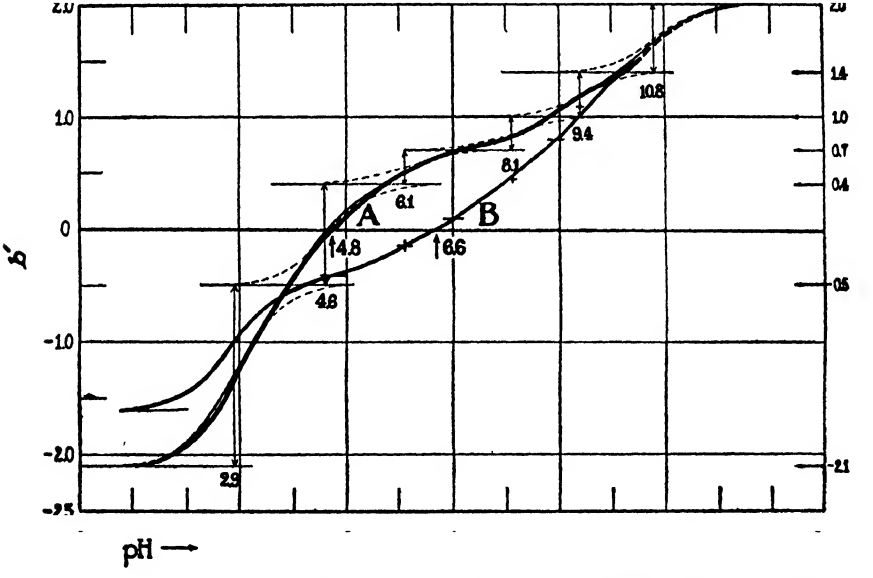

FIG. 2. Egg albumin titration curves. Curve *A*. Experimental curve (heavy line) and "found" curve (light line, see Table IV). Curve *B*. Curve "predicted" from trivalent amino acids.

[8] It would be interesting to know if the discrepancy of 0.5 eq. in the sum of arginine plus 4.6 group in egg albumin is due to an error in the arginine value found on hydrolysis (Column 8). In a subsequent paper it will be shown that the 2.2 eq. of arginine derived from edestin exist as "prearginine."

and egg albumin and that this group is disrupted on hydrolysis. It seems likely that this 4.6 group produces arginine on hydrolysis.

It will be noted that the agreement between the deficiency of arginine and the amount of the 4.6 group is quantitative in the case of gelatin while there is a discrepancy of +0.5 equivalent for egg albumin. Hence we must consider the alternative hypothesis, namely, that the 4.6 group does not produce arginine on hydrolysis and that part of the arginine group in the protein molecule is bound in some unknown manner so that it does not ionize. This seems unlikely, however and does not agree with the data on edestin.[8]

Since the material in proteins which dissociates as a weak base at pH 4.6 appears to give arginine on hydrolysis, we will refer to it as "*prearginine.*"

IV.

Deaminized Gelatin.

Hitchcock[9] showed that the loss of nitrogen on deamination of gelatin agreed with the decrease in acid-combining capacity. It seemed desirable to determine which amino groups in gelatin are removed on deamination. We therefore prepared some deaminized gelatin and titrated it.

If we compare the curve of deaminized gelatin in Fig. 3*A* with that of gelatin it is obvious that the process of deamination has principally removed the free lysine group and that the prearginine, the arginine, and the histidine groups are not materially affected.

In order to analyze the data more accurately we drew the comparison curve,[10] C_1 in Fig. 3*B*. The drop at 10.6 indicates that there is less of the lysine group in the deaminized gelatin. Adding 0.75 equivalent of 10.6 group (and lowering the curve 0.75 equivalent) gives the second comparison curve, C_2.

This second curve (C_2) represents the relation between deaminized gelatin and gelatin deprived of 0.75 equivalent of its lysine group.

[9] Hitchcock, *J. Gen. Physiol.*, 1923–24, vi, 95.

[10] Simms and Levene, *J. Biol. Chem.*, 1926, lxx, 319; Levene and Simms, *J. Biol. Chem.*, 1926, lxx, 327. This method could have been used in analyzing the data in Fig. 1, but was not adopted since the exact pG′ values were uncertain.

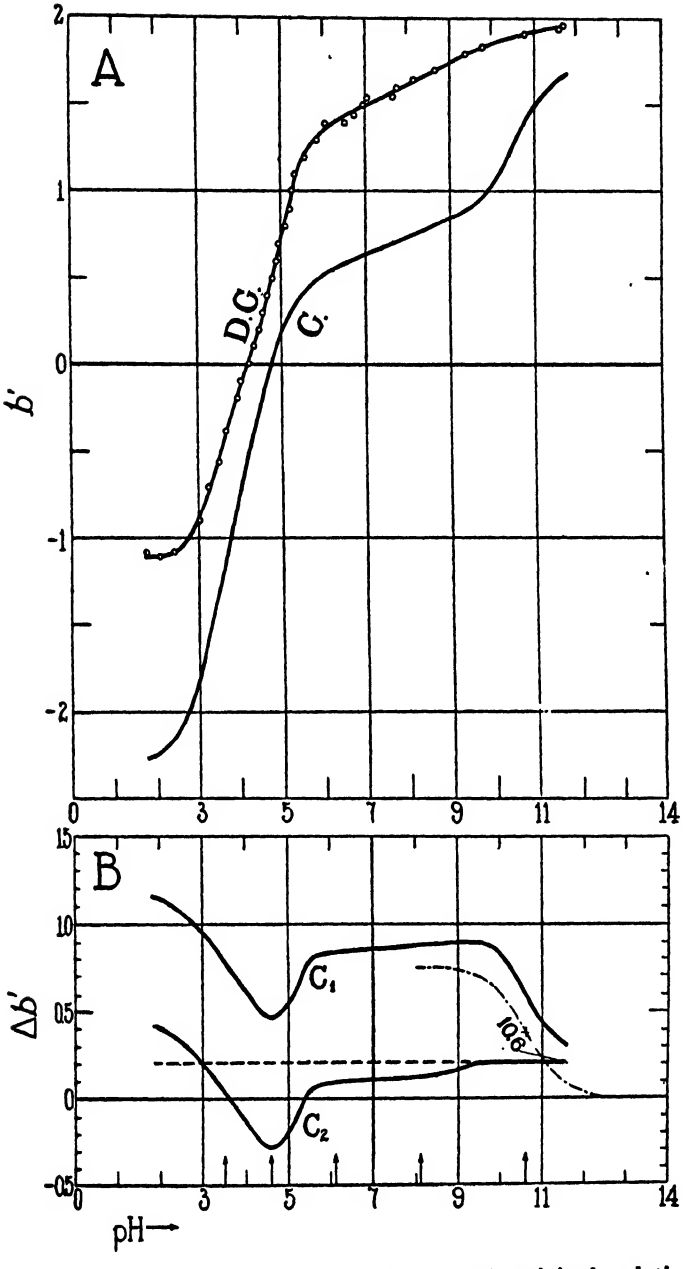

FIG. 3. *A*. Titration curves of gelatin (*G.*) and deaminized gelatin (*D.G.*). *B*. First comparison curve, C_1 (equals *D.G.* minus *G.*); and second comparison curve, C_2 (equals C_1 minus 0.75, plus 0.75 equivalent of 10.6 group).

The drop at 4.6 shows that the index of this group is materially raised (to, say, 5.0 or 5.1) on deamination. There is also a rise of the 6.1 and 8.1 indices and a drop in the acid carboxyl group index (3.5). The height of the curve at pH 11 shows that there is 0.2 equivalent more carboxyl group than before deamination (perhaps due to slight hydrolysis followed by deamination of the amino group produced).

The important points to observe are that *deamination removes a large part of the lysine group* and *does not remove the prearginine, the arginine, or the histidine groups.*

This agrees with the observation of Van Slyke and Birchard[11] that the amino nitrogen of proteins equals half the lysine nitrogen.

TABLE V.

Titration of Deaminized Gelatin, 0.0100 M per 2500 Gm. (2.5 per cent).

pH	$\frac{b-a}{c}$	b'	pH	$\frac{b-a}{c}$	b'
A. More acid solutions.					
1.773	−3.000	−1.083	4.347	.100	.105
2.103	−2.000	−1.105	4.471	.200	.204
2.431	−1.500	−1.081	4.565	.300	.303
3.005	−1.000	−0.889	4.660	.400	.402
3.223	−0.800	− .733	4.802	.500	.502
3.521	− .600	− .567	4.888	.600	.601
3.670	− .400	− .377	4.936	.700	.701
3.962	− .200	− .188	5.101	.800	.801
4.033	− .100	− .090	5.228	.900	.901
4.213	0	+0.006	5.280	1.000	1.001
4.217	0	.006	5.331	1.100	1.101
4.341	0.100	.105			
B. More alkali solutions.					
5.585	1.200	1.200	7.769	1.600	1.600
5.856	1.300	1.300	8.145	1.650	1.650
(6.52)	1.400	1.400	8.648	1.700	1.694
(6.07)	1.400	1.400	9.373	1.800	1.796
6.746	1.450	1.450	9.785	1.850	1.841
6.964	1.500	1.500	10.771	2.000	1.913
(7.07)	1.550	1.550	11.570	2.500	1.944
(7.67)	1.550	1.550	11.693	2.700	1.962

[11] Van Slyke and Birchard, *J. Biol. Chem.*, 1913, xvi, 539.

V.

Hydrolyzed Deaminized Gelatin.

An attempt was made to partially hydrolyze some deaminized gelatin with HCl and determine if the 4.6 group remained intact. The readings were very unsatisfactory and it was not possible to draw any conclusion.

VI.

EXPERIMENTAL.

The data for gelatin are the data without salt in the preceding article of this series.[12] They do not differ materially from the corrected data obtained by others on direct titration of gelatin.

The deaminized gelatin was obtained by treating 100 gm. of isoelectric gelatin in 1 liter of water with 10 gm. of solid $NaNO_2$ and about 15 cc. of glacial acetic acid, heating on steam bath (with occasional stirring) for 4 hours, and then dialyzing through collodion membranes by a method described in another article.[13] The material after 24 hours had a conductivity of 6.4×10^{-5} reciprocal ohms (for 6.7 per cent protein).

This material was titrated by methods previously described.[14] The data are given in Table V.

The experimental curve for egg albumin is taken from the compiled data of Cohn.[5]

VII.

SUMMARY.

Analysis of the experimental titration curves shows that gelatin contains acid groups with dissociation indices at pH 2.9 to 3.5 corresponding quantitatively with the content in dicarboxylic amino acids; and that the acidic group at pH 9.4 in egg albumin agrees with the amount of tyrosine.

[12] Simms, *J. Gen. Physiol.*, 1927–28, xi, 613.

[13] Kunitz and Simms, *J. Gen. Physiol.*, 1927–28, xi, 641.

[14] Simms, *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

The amounts of histidine and lysine present in both these proteins agree quantitatively with basic groups at pH 6.1 and pH 10.4 to 10.6, respectively.

However, the quantity of the arginine group (pH 8.1) in these proteins is considerably less than the amount of arginine found on hydrolysis. This deficiency is compensated (quantitatively with gelatin and approximately with egg albumin) by a basic group at pH 4.6.

The structure of this "4.6 group" should be similar to aniline and cytosine in consisting of an amino group on a conjugated unsaturated (perhaps cyclic) system. It would appear that the 4.6 group is disrupted on hydrolysis, producing arginine, and may be referred to as "prearginine."

The presence of prearginine in proteins, instead of the full amount of arginine, has an important effect on the properties. Otherwise the isoelectric point of gelatin would be 8.0 (instead of 4.7) and of egg albumin 6.6 (instead of 4.8), and the titration curves would be quite different in shape between pH 4 and 10.

Deamination of gelatin produces no decrease in prearginine, arginine, or histidine groups, but removes nearly all of the lysine group.

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THE DISTRIBUTION OF SUGAR BETWEEN BLOOD CORPUSCLES AND BLOOD PLASMA FOR SEVERAL ANIMAL SPECIES.*

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(Received for publication, March 27, 1928.)

A number of investigations to determine the distribution of sugar between blood corpuscles and blood plasma have been performed on human blood. Wu (1), in reviewing this literature and adding to it some of his own analyses, concluded that the blood sugar is about equally divided between blood corpuscles and blood plasma. Wishart (2), in addition to working with human blood, did some work on dog, pony, goat, and sheep blood. She did not determine the per cent of the total sugar that was contained in the blood corpuscles but, by use of her published results as to sugar in corpuscles and plasma together with the corpuscle volumes given in her data, the following averages may be obtained. In dog blood an average of 20.2 per cent of the total blood sugar was in the corpuscles, in pony blood the average was 74.5 per cent, in goat blood it was 12.3 per cent, and in sheep blood it was 47.7 per cent. These figures would indicate that there is a marked species variation as to the distribution of the blood sugar between corpuscles and plasma.

Since work involving the determination of corpuscle and plasma sugar for several additional species was being conducted, it seemed of interest to collect these analyses with the view of noting species differences in distribution.

* Part of the work reported in this paper was done during the summer (1927) at the Iowa Lutheran Hospital, Des Moines, Iowa. I take pleasure in expressing my gratitude to Dr. Harry W. Dahl for the privileges of his laboratory.

TABLE I.

Animal No.	Sugar in corpuscles.	Sugar in plasma.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
			Swine.		
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
380 A	32	125	80	52	19
389 B	46	85	65	51	35
380 B	33	129	88	48	16
388 A	40	95	71	43	24
389 A	39	112	77	48	24
Average.....	38	109	76	48	24
			Guinea pigs.		
1	132	127	130	56	57
2	152	143	148	52	53
3	166	163	164	35	35
4	136	125	130	49	52
5	111	128	120	48	44
Average.....	139	137	138	48	48
			Cattle.		
J 114	29	43	38	41	31
H 55	31	58	46	46	32
5060	29	59	46	45	29
1414	32	60	48	40	27
Average.....	30	55	45	43	30
			Rabbits.		
60	31	83	58	48	25
44	30	127	88	40	14
45	29	102	65	50	22
49	62	114	98	31	19
62	31	129	94	36	12
56	38	141	98	42	16
51	38	107	69	56	31
Average.....	37	115	81	43	20

TABLE I—*Concluded.*

Animal No.	Sugar in corpuscles.	Sugar in plasma.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
			Human.		
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
V. O.	120	104	111	45	48
E. H.	117	115	116	44	45
M. S.	133	116	124	45	48
F. M.	132	105	115	37	42
U. H.	103	91	97	48	51
M. C.	121	96	107	46	52
F. L.	121	91	105	49	56
Average.....	121	103	111	45	49

EXPERIMENTAL.

Sugar determinations on the blood of cattle, swine, rabbits, guinea pigs, and human beings were carried out. The sugar in the blood from humans and guinea pigs was determined by Myers and Bailey's modification of the Lewis-Benedict method (3) with potassium oxalate as the anticoagulant, and that of cattle, swine, and rabbits by the new Folin method (4) with sodium citrate, 2 mg. per cc., as the anticoagulant. Sodium citrate in this amount was found not to alter significantly the results with the new Folin method. While the first method gave much higher values than the second, it was found that the per cent of the total blood sugar in the corpuscles was the same for similar blood samples irrespective of the method employed. The amounts of sugar in corpuscles and plasma were determined directly and the whole blood sugar was calculated. With the exception of those for cattle and swine, the values given represent fasting blood sugars. Blood from the cattle and the swine was drawn between 4 and 5 hours after feeding. The results are given in Table I.

DISCUSSION AND SUMMARY.

The data presented, although limited as to the number of individuals in each species, indicate that the distribution of sugar between blood corpuscles and blood plasma varies with species. That this

variation is not dependent upon corpuscle volume is demonstrated by the relatively constant values obtained for the various species as compared with the values for per cent of total sugar in corpuscles. No explanation for the species difference in sugar distribution is apparent. A possible explanation is suggested by some work of Glassman's (5) in which both free and combined glucose were determined. His data indicate that there is no free glucose in the corpuscles after a triple washing in physiological salt solution. If it is a fact that corpuscles contain no free glucose, then the variations found as regards species may indicate that there is a species difference in the stability of the glucose-protein compound in corpuscles and hence a difference in the degree of hydrolysis resulting when an acid protein precipitant is employed as was the case in the work here reported.

BIBLIOGRAPHY.

1. Wu, H., *J. Biol. Chem.*, 1922, li, 21.
2. Wishart, M. B., *J. Biol. Chem.*, 1920, xliv, 563.
3. Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.
4. Folin, O., *J. Biol. Chem.*, 1926, lxvii, 357.
5. Glassman, B., *Z. physiol. Chem.*, 1925, cl, 16; 1926, clviii, 113.

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THE DISTRIBUTION OF SUGAR BETWEEN CORPUSCLES AND PLASMA IN BLOOD FROM NORMAL HUMAN BEINGS, AND FROM DIABETICS WITH AND WITHOUT INSULIN THERAPY.*

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(Received for publication, March 27, 1928.)

INTRODUCTION.

In a condition characterized by a marked quantitative disturbance in blood sugar as is diabetes mellitus, it would not seem impossible that there might also be a disturbance in the distribution of sugar between corpuscles and plasma. Wu (1), in reviewing the literature and adding to it some of his own analyses, concluded that the blood sugar normally is about equally divided between blood corpuscles and blood plasma in human blood. That this ratio need not be a constant is indicated by a variable distribution of sugar between corpuscles and plasma in blood from different animal species (2). Secker's (3) *in vitro* experiments with insulin suggest that an abnormal distribution of sugar between corpuscles and plasma might be found in diabetes.

However, Wishart (4), in experimental diabetes in dogs, found no marked alteration in the distribution of sugar between corpuscles and plasma, and the distribution observed by Allen, Stillman, and Fitz (5) in the blood of human diabetics under dietary management is not far from that given by Wu for blood from normal individuals.

The following data are published in confirmation of the work of

* The work reported in this paper was done during the summer (1927) at the Iowa Lutheran Hospital, Des Moines, Iowa. I take pleasure in expressing my gratitude to Dr. Harry W. Dahl for the privileges of his laboratory, and to Dr. Edwin B. Winnett for his kindness in obtaining blood samples for me from patients in his practice.

Wishart, and of Allen, Stillman, and Fitz with additional observations concerning the effect of insulin therapy on the blood sugar distribution in diabetics.

EXPERIMENTAL.

Blood was obtained from ten normal individuals, ten diabetics who had never had insulin, and ten diabetics who at the time were on insulin therapy. For the most part it was drawn from the subject after an overnight fasting period. Several of the blood samples,

TABLE I.

Normal Individuals.

Patient No.	Corpuscle sugar.	Plasma sugar.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
1. V. M.	132	101	116	47	54
2. S. E.	123	128	125	50	48
3. U. H.	103	91	97	48	51
4. M. Y.	121	96	107	46	52
5. F. O.	121	91	105	49	56
6. L. E.	163	90	130	55	69
7. M. D.	118	95	104	37	42
8. M. S.	133	116	124	45	48
9. H. B.	117	115	116	44	45
10. B. E.	147	122	130	34	39
Average	126	103	115	45	50

however, were taken between 3 and 4 hours after the subject had eaten.

Corpuscle volume was obtained by centrifugalization in graduated tubes. Myers and Bailey's (6) modification of the Lewis-Benedict method was used in the sugar determinations. Sugar in corpuscles and plasma was determined directly and whole blood sugar calculated from these on the basis of corpuscle volume. In the values given in Tables I to III for per cent of total sugar in corpuscles, the corpuscle volume was used in making the calculations.

TABLE II.

Diabetics Who Have Never Received Insulin.

Patient No.	Corpuscle sugar.	Plasma sugar.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
11. B. N.	155	128	144	58	62
12. M. K.	282	270	274	33	34
13. S. H.	170	182	177	43	41
14. F. Y.	154	141	148	53	55
15. A. R.	130	118	125	58	60
16. Y. I.	213	222	218	44	43
17. L. H.	476	500	486	58	56
18. L. E.	476	571	526	48	43
19. G. G.	206	217	212	49	47
20. C. N.	170	158	164	58	60
Average	243	251	247	50	50

TABLE III.

Diabetics Who Were Receiving Insulin.

Patient No.	Corpuscle sugar.	Plasma sugar.	Whole blood sugar (calculated).	Corpuscle volume.	Per cent of total sugar in corpuscles.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
21. H. H.	400	426	412	52	50
22. M. R.	385	392	389	35	34
23. S. O.	222	233	229	36	39
24. W. R.	426	444	435	48	47
25. L. V.	328	345	337	45	44
26. R. S.	308	244	273	46	51
27. J. S.	263	241	250	42	44
28. M. M.	291	286	287	36	36
29. S. N.	333	314	321	39	40
30. R. E.	168	143	154	45	49
Average	312	307	309	42	44

DISCUSSION.

In accordance with earlier work, no abnormality was found to exist in the distribution of sugar between corpuscles and plasma in diabetic blood. The average amount of sugar in the corpuscles of both normal individuals and diabetics not being treated with insulin was 50 per cent of the total blood sugar. For diabetics who were at the time receiving insulin daily the average amount of sugar in the corpuscles was 44 per cent of the total blood sugar. Mathematically, the values for per cent of sugar in corpuscles for diabetics receiving insulin were found not to vary significantly when compared with similar values for diabetics not receiving insulin therapy or normal individuals.

No correlation was found to exist between the amount of sugar in the corpuscles and the corpuscle volume. There was, however, a very marked correlation between the values for corpuscle sugar and plasma sugar in both of the diabetic series, indicating that there is a free exchange of sugar between corpuscles and plasma. No correlation between values for corpuscle and plasma sugar could be found in the series of normal individuals. This does not mean that in normal blood free exchange of sugar between corpuscles and plasma fails to take place but rather that in blood from a non-diabetic there are a number of factors at work tending to alter the amount of sugar present in either corpuscles or plasma, making a definite correlation unlikely, whereas in diabetic blood there is one factor at work tending to raise the blood sugar and if there is a free exchange of sugar between corpuscles and plasma a definite correlation would be expected.

SUMMARY AND CONCLUSIONS.

1. There is no abnormality in the distribution of sugar between blood corpuscles and blood plasma in diabetic individuals.

2. Insulin therapy causes no significant alteration in distribution of sugar between the blood corpuscles and blood plasma.

3. The correlation found to exist between blood corpuscle sugar and blood plasma sugar in diabetics indicates that there is a free exchange of sugar between corpuscles and plasma.

BIBLIOGRAPHY.

1. Wu, H., *J. Biol. Chem.*, 1922, li, 21.
2. Shope, R. E., 1928, lxxviii, 107.
3. Secker, J., *J. Physiol.*, 1925, lx, 286.
4. Wishart, M. B., *J. Biol. Chem.*, 1920, xliv, 563.
5. Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monographs of The Rockefeller Institute for Medical Research, No. 11, New York, 1919.
6. Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

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